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(54) Title: COMPOSITIONS FACILITATING DRUG PENETRATION AND DRUG RETENTION IN SKIN

(57) Abstract: The present invention relates to delivery systems comprising a saturating agent and a lipophilic or hydrophilic agent in solution in a polar solvent, a surfactant or an organic solvent and methods of use thereof. The systems and methods of this invention find application in transdermal drug delivery, for the treatment of local and systemic diseases, disorders and/or conditions.



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## COMPOSITIONS FACILITATING DRUG PENETRATION AND DRUG RETENTION IN SKIN

### FIELD OF THE INVENTION

The present invention is directed to transdermal delivery systems comprising a saturating agent and a compound in solution in a polar solvent, a surfactant or an organic solvent and methods of use thereof. The compound in solution may be lipophilic or hydrophilic. The systems and methods of this invention find application in the treatment of local and systemic diseases, disorders and/or conditions.

### BACKGROUND OF THE INVENTION

Topical application and retention of a variety of active ingredients is an effective means of providing skin care, finding use in cosmetics and in treating, *inter-alia*, skin diseases (fungal, viral, and parasitic infections, inflammatory syndromes, tumors). Drug retention within the skin, however, may be hampered, as the skin may present a formidable barrier to efficient absorption.

Many active agents, such as proteins or peptides, have a high molecular weight, yet the skin is an extremely effective barrier that is highly impermeable to such large molecular weight molecules. Drugs with high lipid solubility may pass easily through the membrane, while those with low lipid solubility need a membrane carrier or active transport system. The formulation and delivery of these molecules in the skin is challenging. The partition coefficient of a drug depends upon both polarity and size. Drugs with high dipole moment, even though unionized, have low lipid solubility and, hence, penetrate poorly.

Further confounding drug delivery in the skin is the fact that methods commonly used to overcome the skin barrier facilitate drug penetration, and not retention. Thus, drug retention within the skin, and within specific layers of the skin, is as yet, not amenable to much control.

### SUMMARY OF THE INVENTION

In one embodiment, this invention provides a topical delivery system for skin retention of a lipophilic agent, the system comprising:

a saturating agent; and

a lipophilic agent in a vehicle, wherein said vehicle comprises a polar solvent, a surfactant, an organic solvent or a combination thereof;

5 According to this aspect of the invention, and in one embodiment, the saturating agent is a polar solvent, an organic solvent or a surfactant, and in another embodiment, has a molecular weight of up to 1450 Da .

In one embodiment, the organic solvent is isopropyl myristate. In another embodiment, the  
10 surfactant is a Tween, a Span or 1,2-octanediol. In another embodiment, the polar solvent is water.

In another embodiment, the saturating agent is a surfactant, or, in another embodiment, the saturating agent is an organic solvent and the vehicle is water, or, in another embodiment, the vehicle is a surfactant. In another embodiment, the saturating agent is a Span or a Tween and the vehicle is 1,2-  
15 octanediol, or in another embodiment, the saturating agent is 1,2-octanediol and the vehicle is a Span or Tween. In another embodiment, the saturating agent is an organic solvent and said vehicle is a surfactant, or in another embodiment, the saturating agent is a surfactant and the vehicle is an organic solvent.

20 In another embodiment, this invention provides a topical delivery system for lipophilic agent permeation of the skin, comprising a saturating agent and a lipophilic agent in solution in an organic solvent or a surfactant. In one embodiment, the saturating agent is a polar solvent, an organic solvent or a surfactant, which, in another embodiment has a molecular weight of at least 18 Da. In another embodiment, the vehicle has a molecular weight of at least 18 Da.

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In one embodiment, the saturating agent is a water or Tween and the vehicle is isopropyl myristate. In another embodiment, saturating agent is isopropyl myristate or Span and the vehicle is Tween. In another embodiment, the saturating agent is Tween and the vehicle is Span.

30 In another embodiment, the lipophilic agent is retinoic acid.

In another embodiment, this invention provides a topical delivery system, for retention of a hydrophilic agent, comprising a saturating agent and a hydrophilic drug in solution in a polar solvent, a surfactant or an organic solvent. In one embodiment, the saturating agent is a polar solvent, an

organic solvent or a surfactant, and in another embodiment, has a molecular weight of up to 1450 Da. In another embodiment, the vehicle has a molecular weight of up to 1450 Da.

According to this aspect of the invention and in another embodiment, the saturating agent is an organic solvent, a surfactant, or a polar solvent, and the vehicle is water. In another embodiment, the saturating agent is an organic solvent and the vehicle is a surfactant. In another embodiment, the saturating agent is a surfactant and the vehicle is a surfactant. According to this aspect of the invention, and in one embodiment, the saturating agent is a Tween or 1,2-octanediol and the vehicle is a Span. In another embodiment, the saturating agent is a Tween or a Span and the vehicle is 1, 2-octanediol. In another embodiment, the saturating agent is a surfactant and the vehicle is an organic solvent. In another embodiment, the saturating agent is an organic solvent and the vehicle is a surfactant.

In another embodiment, this invention provides a topical delivery system for hydrophilic agent permeation of the skin, comprising a saturating agent and a hydrophilic agent in solution in an organic solvent or a surfactant. In one embodiment, the saturating agent is a polar solvent, an organic solvent or a surfactant, which in another embodiment, has a molecular weight of at least 18 Da. In another embodiment, the vehicle has a molecular weight of at least 18 Da.

According to this aspect of the invention, and in one embodiment, the saturating agent is a water or Tween and the vehicle is isopropyl myristate. In another embodiment, the saturating agent is isopropyl myristate or Span and the vehicle is Tween. In another embodiment, the saturating agent is Tween and the vehicle is Span.

In another embodiment, the hydrophilic agent is mannitol.

In another embodiment of this invention, the delivery systems of this invention are formulated for delivery from a two-chamber liquid-patch.

In another embodiment, this invention provides a method of transdermal drug delivery in a subject, comprising contacting a skin surface of said subject with a saturating agent and contacting the skin surface with a drug in a vehicle, wherein the vehicle comprises a polar solvent, a surfactant, an organic solvent or a combination thereof and whereby the drug is retained within, or permeates a skin in the subject, thereby being a method of transdermal drug delivery in the subject.

In one embodiment, the drug is lipophilic, or in another embodiment, hydrophilic. In one embodiment, the drug treats a condition of the skin, nails, scalp, hands, feet, or combination thereof in the subject. In another embodiment, the drug treats infection, inflammation, eczema, dermatitis, keratosis, urticaria, allergy, acne, folliculitis, furuncles, psoriasis, rosacea, pityriasis, cancer, precancerous lesions, muscular pain, arthritis, heart disease, osteoporosis, osteopetrosis, burns, non-healing wounds, scars, skin ulcers, hyperhidrosis, ichthyosis, lupus of the skin, sun damage, vitiligo, or a combination thereof, in the subject. In another embodiment, the drug is an analgesic, an anesthetic, an antioxidant, a growth factor, a hormone, an extracellular matrix component, or a combination thereof.

In one embodiment, the drug is formulated as a microemulsion. In another embodiment, the saturating agent and the vehicle comprising a drug are formulated for delivery from a two-chamber liquid-patch.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a ternary phase diagram of microemulsion system and phase characterization

Figure 2 schematically depicts the instrument and experimental set-up for evaluating skin barrier integrity. A: Components of the instrument for measuring resistivity: FG: function generator (sine wave: 100 mV PP, 1kHz and 10Hz); M: multimeter (measuring currents of  $\mu\text{A}$ ); E: Ag-AgCl electrodes; D: donor cell; S: human cadaver-full thickness skin (hc-FTS); R: receiver cell. Ion passage through the skin is measured, with skin barrier integrity considered as a function of resistivity, which is extrapolated mathematically by measured currents. B: Experimental setup of the diffusion experiments performed. Samples were placed in diffusion cells at conditions of  $\Phi$ : 0.636 cm<sup>2</sup> in a 2.4 mL total volume. Skin samples were pretreated for 12 hours (saturation) with the microemulsions without drugs or for 1 hour (hydration) with PBS. 2 mL of drug loaded vehicle (1  $\mu\text{Ci/g}$  vehicle) was added per sample, and allowed to permeate in skin for 18 hours. Skin resistivity was evaluated at t-12, t0 and t18, and drug was quantified in a scintillation counter.

Figure 3 demonstrates trans retinoic acid penetration parameters through the skin using microemulsions. A. Skin resistivity in response to treatment. Skin resistivity was extrapolated at the beginning (t 0) and at the end (t 18) of the permeation experiments using Ohm's law. Values are

given in  $k\Omega cm^2$ . B. Comparison of the total amount of RA retained and permeated throughout human cadaver abdominal full-thickness skin. Values are given in 'disintegration per minutes' (DPM); 'Perm' bars indicate the amounts of RA that permeated through the skin; 'Ret' bars indicate the amounts of RA that was retained in the skin. C. Difference in current flow through the skin. Reported currents were calculated as  $\Delta I = I(0\text{ h}) - I(18\text{ h})$  and are given in  $\mu A$ . D. Trans retinoic acid permeation kinetics. Differences in the cumulative permeated trans retinoic acid amounts as a function of time (hours) and vehicles (microemulsions). Values are given in 'disintegration per minutes' (DPM). E. Comparison of the total amount of trans retinoic acid retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts.

Figure 4. Mannitol penetration parameters through the skin using microemulsions. A. Skin resistivity was extrapolated as in Figure 3. B. Variation of mannitol permeation parameters as a function of the hydrophilic content of each microemulsion. R = total amount of mannitol retained in the skin; R/P = ratio of mannitol total amounts retained and permeated through the skin; R+P= sum of the mannitol amounts retained and permeated through the skin; P= total amount of mannitol permeated through the skin. C. Difference in current flow through the skin. Reported currents were calculated as in Figure 3. D Mannitol permeation kinetics. Differences in the cumulative permeated mannitol amounts as a function of time (hours) and vehicles (microemulsions). E. Comparison of the total amount of mannitol retained and permeated throughout human cadaver abdominal full-thickness skin.

Figure 5 demonstrates trans retinoic acid penetration parameters through the skin when delivered in particular microemulsion components. Vehicles used to deliver trans retinoic acid through the skin were water (W), 1,2-octanediol (O), Tween 80 (T), Span 80 (S), and isopropyl myristate (I). A. Skin resistivity was extrapolated at the beginning (t 0) and at the end (t 18) of the permeation experiments using Ohm's law. Values are given in  $k\Omega cm^2$ . B. Comparison of the total amount of RA retained and permeated throughout human cadaver abdominal full-thickness skin. Values are given in 'disintegration per minutes' (DPM); 'Perm' bars indicate the amounts of RA that permeated through the skin; 'Ret' bars indicate the amounts of RA that was retained in the skin. C. Difference in current flow through the skin. Reported currents were calculated as  $\Delta I = I(0\text{ h}) - I(18\text{ h})$  and are given in  $\mu A$ . D. Trans retinoic acid permeation kinetics. Differences in the cumulative permeated trans retinoic acid amounts as a function of time (hours) and vehicles (microemulsion components). Values are

given in 'disintegration per minutes' (DPM). E. Comparison of the total amount of trans retinoic acid retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts.

5 Figure 6 demonstrates the relationship between the log value for the delivery vehicle molecular weight and: the log value for the total amount of 3H-RA that entered in the skin (R+P); the total amount of RA retained within the skin; the total amount of RA that permeated through the skin; and the RA R/P values. Data refers to PBS-hydrated (1h) skin samples. In each graph, vehicles are positioned from the left to the right in the following order: W, O, I, S, and T, which is the order of  
10 the increasing Log MW.

Figure 7. Mannitol penetration parameters through the skin when delivered in particular microemulsion components. Vehicles used to deliver trans retinoic acid through the skin were water (W), 1,2-octanediol (O), Tween 80 (T), Span 80 (S), and isopropyl myristate (I). A. Skin resistivity  
15 was extrapolated as in Figure 5. B. Difference in current flow through the skin. Reported currents were calculated as in C. Mannitol permeation kinetics. Differences in the cumulative permeated mannitol amounts as a function of time (hours) and vehicles (microemulsion components). Values are given in 'disintegration per minutes' (DPM). D. Comparison of the total amount of mannitol retained and permeated throughout human cadaver abdominal full-thickness skin.

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Figure 8 demonstrates the relationship between the log value for the delivery vehicle molecular weight and: the log value for the total amount of MN that entered in the skin (R+P); the total amount of MN retained within the skin; the total amount of MN that permeated through the skin; and the MN R/P values. Data refers to PBS-hydrated (1h) skin samples. In each graph, vehicles are positioned  
25 from the left to the right in the following order: W, O, I, S, and T, which is the order of the increasing Log MW.

Figure 9 demonstrates trans retinoic acid penetration through skin when delivered in particular microemulsion components, in skin samples saturated with water. Vehicles used to deliver trans  
30 retinoic acid through the skin were water (W), 1,2-octanediol (O), Tween 80 (T), Span 80 (S), and isopropyl myristate (I). Skin samples were hydrated for 12 hours with water prior to start the permeation experiments. A. Skin resistivity extrapolated at the beginning of the saturation phase ( $t_{-12}$ ), at the beginning of the permeation experiments ( $t_0$ ) and at the end ( $t_{18}$ ) of the permeation experiments using Ohm's law. Values are given in  $k\Omega\text{cm}^2$ . B. Comparison of the total amount of RA

retained and permeated throughout human cadaver abdominal full-thickness skin. Values are given in 'disintegration per minutes' (DPM); 'Perm' bars indicate the amounts of RA that permeated through the skin; 'Ret' bars indicate the amounts of RA that was retained in the skin. C. Difference in current flow through the skin. Reported currents were calculated as  $\Delta I = I(t-12) - I(t-0)$  and as  $\Delta I = I(t-0) - I(t-18)$ , and are given in  $\mu A$ . D. Trans retinoic acid permeation kinetics. Differences in the cumulative permeated trans retinoic acid amounts as a function of time (hours) and vehicles (microemulsion components). Values are given in 'disintegration per minutes' (DPM). E. Comparison of the total amount of trans retinoic acid retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts.

Figure 10. Relationship between the log value for the delivery vehicle molecular weight and: the log value for the total amount of RA that entered in the skin (R+P); the total amount of RA that was retained within the skin; the total amount of RA that permeated through the skin; and the RA R/P values. Data refers to water-saturated (12 h) skin samples. In each graph, vehicles are positioned from the left to the right in the following order: W, O, I, S, and T, that is the order of the increasing Log MW.

Figure 11 demonstrates mannitol penetration parameters through the skin using microemulsion components and skin samples saturated with water. Vehicles used to deliver mannitol through the skin were water (W), 1,2-octanediol (O), Tween 80 (T), Span 80 (S), and isopropyl myristate (I). Skin samples were hydrated for 12 hours with water prior to start the permeation experiments. A. Skin resistivity was extrapolated as described in Figure 9. B. Difference in current flow through the skin. Reported currents were calculated as described. C. Mannitol permeation kinetics. Differences in the cumulative permeated mannitol amounts as a function of time (hours) and vehicles (microemulsion components). D. Comparison of the total amount of mannitol retained and permeated throughout human cadaver abdominal full-thickness skin.

Figure 12 demonstrates the relationship between the log value of the delivery vehicle molecular weight and: the log value for the total amount of MN that entered in the skin (R+P); the total amount of MN that was retained within the skin; the total amount of MN that permeated through the skin; and the MN R/P values. Data refers to water-saturated (12 h) skin samples. In each graph, vehicles



are positioned from the left to the right in the following order: W, O, I, S, and T, that is the order of the increasing Log MW.

Figure 13 demonstrates trans retinoic acid penetration parameters in skin when water is the delivery vehicle and skin samples were saturated with microemulsion components. Skin samples were saturated with microemulsion components [water (W), 1,2-octanediol (O), Tween 80 (T), Span 80 (S), and isopropyl myristate (I)] for 12 hours prior to start the permeation experiments. In some figures values of W (PBS-hydrated skin) are introduced for comparative purposes. A. Skin resistivity was extrapolated at the beginning of the saturation phase as described. B. Comparison of the total amount of RA retained and permeated throughout human cadaver abdominal full-thickness skin. C. Difference in current flow through the skin. Reported currents were calculated as described. D. Trans retinoic acid permeation kinetics. Differences in the cumulative permeated trans retinoic acid amounts as a function of time (hours) and skin saturating vehicles (microemulsion component). Values are given in 'disintegration per minutes' (DPM). E. Relationship between the log value of the delivery vehicle molecular weight and: the log value for the total amount of RA that entered in the skin (R+P); the total amount of RA that was retained within the skin; the total amount of RA that permeated through the skin; and the RA R/P values. Data refers to microemulsion components-saturated (12 h) skin samples; drug delivery vehicle was water. In each graph, skin saturating vehicles are positioned from the left to the right in the following order: W, O, I, S, and T, which is the order of the increasing Log MW. Symbols in blue represent the corresponding values found for W.

Figure 14 demonstrates mannitol penetration parameters through skin saturated with microemulsion components, when water is the delivery vehicle. Skin samples were saturated with microemulsion components, as above. A. Skin resistivity was extrapolated as previously described. B. Difference in current flow through the skin. Reported currents were calculated as described. C. Mannitol permeation kinetics. Differences in the cumulative permeated mannitol amounts as a function of time (hours) and skin saturating vehicles (microemulsion component). D. Comparison of the total amount of mannitol retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts.

Figure 15 demonstrates the relationship between log value for the vehicle molecular weight and: the log of the total amount of MN that entered in the skin (R+P); the total amount of MN that was retained within the skin; the total amount of MN that permeated through the skin; and the MN R/P

values. Data refers to microemulsion component-saturated (12 h) skin samples, followed by drug delivery in water. In each graph, skin was saturated with a vehicle, which is represented in the graph from left to the right in the following order: W, O, I, S, and T, which represents the order of increasing Log MW of the vehicle.

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Figure 16 demonstrates trans retinoic acid penetration parameters through the skin using microemulsion components as the delivering vehicles and skin samples saturated with isopropyl myristate. Skin samples were saturated with microemulsion components [water (W), 1,2-octanediol (O), Tween 80 (T), Span 80 (S), and isopropyl myristate (I)] for 12 hours prior to start the permeation experiments. A. Skin resistivity was extrapolated as indicated in Figure 6. B. Comparison of the total amount of RA retained and permeated throughout human cadaver abdominal full-thickness skin. Values are given in 'disintegration per minutes' (DPM); 'Perm' bars indicate the amounts of RA that permeated through the skin; 'Ret' bars indicate the amounts of RA that was retained in the skin. C. Differences in current flow through the skin were calculated as indicated above. D. Trans retinoic acid permeation kinetics. Differences in the cumulative permeated trans retinoic acid amounts as a function of time (hours) and skin saturating vehicles (I). Values are given in 'disintegration per minute' (DPM). E. Comparison of the total amount of trans retinoic acid retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts. F. Relationship between the log value for the delivery vehicle molecular weight and: the log value for the total amount of RA that entered in the skin (R+P); the total amount of RA that was retained within the skin; the total amount of RA that permeated through the skin; and the RA R/P values. Data refers to isopropyl myristate-saturated (12 h) skin samples; drug delivering vehicles were the microemulsion components. In each graph, skin delivering vehicles are positioned from the left to the right in the following order: W, O, I, S, and T, that is the order of the increasing Log MW.

Figure 17 demonstrates a comparison of trans retinoic acid R/P ratios using microemulsion components as delivering vehicles and differently pre-treated skin samples. For each group of columns, from the left to the right, skin samples where hydrated with PBS, saturated with water or with isopropyl myristate.

Figure 18 demonstrates mannitol penetration parameters through the skin when microemulsion components are the respective delivery vehicle and skin samples are saturated with isopropyl myristate. A. Skin resistivity was extrapolated as described. B. Difference in current flow through

the skin. Reported currents were calculated as described. C. Mannitol permeation kinetics. Differences in the cumulative permeated mannitol amounts as a function of time (hours) and skin saturating vehicles (I). Values are given in 'disintegration per minutes' (DPM). D. Comparison of the total amount of mannitol retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts. E. Relationship between the log value for the vehicular molecular weight and: the log value for the total amount of MN that entered in the skin (R+P); the total amount of MN that was retained within the skin; total amount of MN that permeated through the skin; and the MN R/P values. Data refers to isopropyl myristate-saturated (12 h) skin samples; drug delivering vehicles were the microemulsion components. In each graph, skin delivering vehicles are positioned from the left to the right in the following order: W, O, I, S, and T, which is the order of the increasing Log MW.

Figure 19 demonstrates trans retinoic acid penetration parameters through skin when isopropyl myristate is the delivery vehicle and skin samples are saturated with microemulsion components. A. Skin resistivity was extrapolated as described. B. Comparison of the total amount of RA retained and permeated throughout human cadaver abdominal full-thickness skin. C. Difference in current flow through the skin. Reported currents were calculated as described. D. Trans retinoic acid permeation kinetics. Differences in the cumulative permeated trans retinoic acid amounts as a function of time (hours) and skin saturating vehicles (microemulsion components). Values are given in 'disintegration per minutes' (DPM). E. Comparison of the total amount of trans retinoic acid retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts. F. Relationship between the log value for vehicular molecular weight and: the log value for the total amount of RA that entered in the skin (R+P); the total amount of RA that was retained within the skin; the total amount of RA that permeated through the skin; and the RA R/P values. Data refers to microemulsion components-saturated (12 h) skin samples; drug delivery vehicle was isopropyl myristate. In each graph, skin delivering vehicles are positioned from the left to the right in the following order: W, O, I, S, and T, which is the order of increasing Log MW.

Figure 20 demonstrates mannitol penetration parameters through the skin when isopropyl myristate is the delivery vehicle and skin samples are saturated with microemulsion components. A. Skin resistivity was extrapolated as described. B. Difference in current flow through the skin. Reported currents were calculated as described. C. Mannitol permeation kinetics. Differences in the

cumulative permeated mannitol amounts as a function of time (hours) and skin saturating vehicles (microemulsion components). Values are given in 'disintegration per minutes' (DPM). D. Comparison of the total amount of mannitol retained and permeated throughout human cadaver abdominal full-thickness skin; the comparison is expressed as a ratio 'R/P' where R = retained and P = permeated amounts. E. Relationship between the log of delivering vehicle molecular weight and the log of (left-up) the total amount of MN that entered in the skin (R+P); (left-down) the total amount of MN that was retained within the skin; (right-up) the total amount of MN that permeated through the skin; (right-down) the MN R/P values. Data refers to isopropyl myristate-saturated (12 h) skin samples; drug delivering vehicles were the microemulsion components. In each graph, skin delivering vehicles are positioned from the left to the right in the following order: W, O, I, S, and T, that is the order of the increasing Log MW.

Figure 21 demonstrates trans retinoic acid penetration parameters through the skin using microemulsion surfactant components as delivery vehicles in skin samples saturated with microemulsion surfactant components. Skin samples were saturated with [1,2-octanediol (O), Tween 80 (T), Span 80 (S)], for 12 hours prior to start the permeation experiments. A. Skin resistivity was extrapolated as described. B. Comparison of the total amount of RA retained and permeated throughout human cadaver abdominal full-thickness skin. C. Difference in current flow through the skin. Reported currents were calculated as described. D. Trans retinoic acid permeation kinetics. E. Comparison of the total amount of trans retinoic acid retained in and permeated throughout human cadaver abdominal full-thickness skin.

Figure 22 demonstrates mannitol penetration parameters through the skin using microemulsion surfactant components as the delivery vehicles and skin samples saturated with microemulsion surfactant components. Skin samples were saturated with 1,2-octanediol (O), Tween 80 (T), Span 80 (S), for 12 hours prior to start the permeation experiments. A. Skin resistivity was extrapolated as described. B. Difference in current flow through the skin. Reported currents were as described. C. Mannitol permeation kinetics. Differences in the cumulative permeated mannitol amounts as a function of time (hours) and skin saturating vehicles (microemulsion surfactant components). Values are given in 'disintegration per minutes' (DPM). D. Comparison of the total amount of mannitol retained in and permeated throughout human cadaver abdominal full-thickness skin.

Figure 23 demonstrates SPION size and size distribution. TEM micrographs of round-shaped (A) TMAOH- and (B) AOT-SPIONs. The grey shadows in fig. 23B are produced by excess AOT in AOT-SPION dispersions. DLS measurements revealed that both nanoparticles formed various reversible floccules when dispersed in water. However, TMAOH-SPION (C) dispersion showed a greater heterogeneity in floccule size than AOT-SPION one (D).

Figure 24 demonstrates skin electrical resistivity of specimens exposed to (A) TMAOH- and (B) AOT- SPIONs. Skin resistivity was extrapolated using Ohm's law from current measurements at 1 kHz and 10 Hz along the time point of permeation experiments. Abbreviation in legends: Bl: Blank control solution; W: PBS control solution; Np: nanoparticle dispersion. Please, find further explanation in experimental section under 'diffusion experiments'.

Figure 25 presents light transmission microscope (LTM) images of SPION treated skin specimens. LTM images of toluidine-stained 2  $\mu\text{m}$  sections (A-C) showed good skin morphology. Viable epidermis was particularly compact in PBS-treated specimens (A); in here it is also possible to distinguish protective surface lipids (indicated with red circles). Desmosomes of viable epidermis were clearly distinguishable in TMAOH- (B) and AOT-SPION (C) treated skin samples. In this latter case, profound gaps between epidermal cells (indicated with red circles in C) were also very commonly observed. LTM images of hematoxylin-stained 10  $\mu\text{m}$  sections are presented in D-E. SPION deposits are well visible within the SC layers of sections belonging to skin samples preventively hydrated with PBS for 24 hr prior to start diffusion experiments (D). Deposits decreased their dimensions proceeding toward the SC interior. Sometimes, a fine brown granularity was observed in the upper layers of viable epidermis, as indicated by the yellow circle in E. This granularity was easily distinguishable from melanin deposits that, instead, are commonly found in the basal layer of epidermis (yellow asterisks in E).

Figure 26 shows energy dispersion spectrometry-Scanning electron microscope (EDS-SEM) images from backscattered electrons of SPION treated skin samples. Image A shows a thick sample where it is possible to identify SC, viable epidermis, dermis, and a hair follicle. The area surrounded by the green squared has been magnified and is shown in image C. In here, many whitish aggregates are clearly visible. Some of them (those in the white squares) were analyzed for their chemical composition, and a typical resulting graph is proposed in image D. Presence of Fe K $\alpha$ 1 signal, Fe highest peak, makes unquestionable that those white masses are made of Fe. Please note that image

C was taken within the epidermis, being SC and viable epidermis thickness of 10-20  $\mu\text{m}$  and 50-100  $\mu\text{m}$ , respectively. The asterisked white squared (image C) is the air follicle opening. Image B reveals that SPIONs (white spots) were commonly found within the epidermis. In addition, the low magnification of this image shows that specimens suffered a fracture at the epidermis-dermis junction. This fracture was observed in all specimens and is well displayed in image E where is also possible to see typical holes of dermis structure. Accumulation within the dermis was very rare, an example of what was found is exhibited in image G. Finally, images F and H represent the chemical composition of white areas within the squares in E and G, respectively. Readers may note that chemical composition of aggregates was consistent regardless skin region.

### **DETAILED DESCRIPTION OF THE PRESENT INVENTION**

The present invention is directed, in some embodiments, to delivery systems comprising a saturating agent and a compound in solution in a polar solvent, a surfactant or an organic solvent and methods of use thereof.

In one embodiment, this invention provides a topical delivery system for skin retention of a lipophilic agent, the system comprising:

- a saturating agent; and
- a lipophilic agent in a vehicle, wherein said vehicle comprises a polar solvent, a surfactant, an organic solvent or a combination thereof;

The systems and methods of this invention are for delivery to or across a skin of a subject. In one embodiment, the term "skin," as used herein, refers to the epidermis and/or dermis of an animal. Mammalian skin consists of two major, distinct layers. The outer layer of the skin is called the epidermis. The epidermis is comprised of the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale, with the stratum corneum being at the surface of the skin and the stratum basale being the deepest portion of the epidermis. The epidermis is between 50  $\mu\text{m}$  and 0.2 mm thick, depending on its location on the body.

Beneath the epidermis is the dermis, which is significantly thicker than the epidermis. The dermis is primarily composed of collagen in the form of fibrous bundles. The collagenous bundles provide

support for, inter alia, blood vessels, lymph capillaries, glands, nerve endings and immunologically active cells.

One of the major functions of the skin as an organ is to regulate the entry of substances into the body. The principal permeability barrier of the skin is provided by the stratum corneum, which is formed from many layers of cells in various states of differentiation. The spaces between cells in the stratum corneum is filled with different lipids arranged in lattice-like formations which provide seals to further enhance the skin's permeability barrier.

The permeability barrier provided by the skin is such that it is largely impermeable to molecules having molecular weight greater than about 750 Da. For larger molecules to cross the skin's permeability barrier, mechanisms other than normal osmosis must be used. Consequently, there is a need for compositions and methods to facilitate the transport of molecules through the skin's permeability barrier to the epidermis and the dermis.

Once the molecules cross the skin permeability barrier, they may be retained within the skin, which, in one embodiment, refers to any layer of the skin, as described herein, or the molecule may exit the skin and access other regions of the body, a process which, in one embodiment, is referred to herein as permeation. In another embodiment, the term "permeation" may refer to the molecule accessing other layers of the skin which are beneath the stratum corneum, and the term may reflect a relative relationship, in another embodiment, to that of "retention", wherein the latter is present at a greater concentration, or in another embodiment, for a longer period of time, in a particular region of a skin, such as, for example, within a particular stratum, or in another embodiment, within the epidermis, or in another embodiment, within the dermis. Relative changes in the pattern of deposition of the molecule, then refers, in another embodiment, to "permeation".

In one embodiment, this invention provides for retention of a hydrophilic or lipophilic molecule in skin, where the molecule is prepared as part of a microemulsion. In one embodiment, the microemulsion is as set forth herein in Table 1.

Emulsions, in general, are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o)

emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active agent, which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed.

In one embodiment of this invention, the systems and methods for delivery make use of microemulsions, or components thereof. In one embodiment, the term "microemulsion" refers to a system that is prepared by dispersing an oil in an aqueous surfactant solution and adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Microemulsions are thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

In one embodiment, the delivery systems of this invention comprise saturating agent, which, in one embodiment is a surfactant, a polar solvent or an organic solvent.

In one embodiment, the terms "surfactants" "surface active agents" and "co-surfactants" are to be considered synonymous, and may be used in the systems and methods of this invention. Surfactants



have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Surfactants used in the systems and methods of this invention may include, but are not limited to, ionic surfactants, nonionic surfactants, Tween, including Tween 20, 40, 60 or 80, Span, including Span 80, Brij 96, Emulsifying Wax NF, Glyceryl Stearate, Glyceryl Stearate SE, Glycol Stearate, Glycol Stearate SE, Glycereth-20 Stearate, Glyceryl Behenate, Glyceryl Hydroxystearate, Glyceryl Laurate SE, Glyceryl Oleate, Glyceryl Oleate SE, Propylene Glycol Oleate, Propylene Glycol Oleate SE, Propylene Glycol Stearate, Propylene Glycol Stearate SE, Sorbitan Stearate, Sorbitan Trioleate, water dispersible metal soaps (Sodium Stearate), Behenyl Dimethicone Copolymers, Lauryl Methicone Copolymers, Cetyl Methicone Copolymers, Cetyl Dimethicone Copolymers, Stearyl Dimethicone Copolymers, Dimethicone Copolymers and mixtures thereof. polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. In one embodiment, the term "cosurfactant" may refer to an alcohol or diol such as but not limited to 1,2-octanediol, ethanol, 1-propanol, and 1-butanol, which, in another embodiment, may serve to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. It is to be understood that any surfactant or cosurfactant may be incorporated in the systems and for use in the methods of this invention, as may any combination thereof.

The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol.

The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

5

In one embodiment, the systems and methods of this invention make use of a polar solvent, which, in one embodiment, will have a Snyder polarity index of greater than 2.5. In one embodiment, the polar solvent may comprise an alcohol, such as methanol, isopropanol, n-propanol, or ethanol. In one embodiment, the polar solvent may be water. In another embodiment, the polar solvent may be an ether, such as diethylether or methyl tertiary butyl ether (MTBE). In another embodiment, the polar solvent may be acetone, acetonitrile or ethylacetate.

In one embodiment, the systems and methods of this invention make use of an organic solvent. In one embodiment, the term "organic solvent" may refer, *inter-alia*, to any non-toxic solvent and may assist, in another embodiment, in the solubilization of the agents of this invention. In one embodiment, "organic solvent" refers to molecules containing at least one carbon atom, and which is a liquid at 25° C. Examples of organic solvents include, *inter-alia*, alcohols, like, methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol and 2-methyl propan-1-ol; ethers, like diethyl ether and methyl t-butyl ether; N,N-dialkyl amides, like N,N-dimethylformamide and N,N-dimethylacetamide; ketones, like acetone and butanone; dialkyl sulfoxides, like dimethyl sulfoxide; isopropyl esters, like isopropyl myristate or isopropyl palmitate.

20

In one embodiment, a "water-in-oil" type microemulsion (with oil as the continuous phase) comprising the desired compound is prepared. In one embodiment, the term "continuous phase" refers to the external phase, as compared to the "dispersed phase", which is the internal phase.

25

According to this aspect of the invention, and in one embodiment, the water or dispersed phase comprises a dispersion of a water soluble compound of interest. In one embodiment, the "external" oil phase of the microemulsion may also include a release rate modifying agent, or in another embodiment, a compound of interest. It is to be understood that incorporation of a compound of interest within a particular microemulsion, or component/s thereof will reflect the physico-chemical properties of the same, of the compound of interest, or a combination thereof, and will be understood by those skilled in the art.

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In another embodiment, an "oil-in-water" type microemulsion (with water as the continuous phase) comprising the desired compound is prepared. In one embodiment, the term "continuous phase" refers to the external phase, as compared to the "dispersed phase", which is the internal phase.

- 5 According to this aspect of the invention, and in one embodiment, the water or continuous phase comprises a water soluble compound of interest, or in another embodiment, the dispersed phase may comprise a compound of interest, which, in another embodiment, is lipophilic.

10 In one embodiment, oil phase microdroplets may be formed *in situ* by heating a polymerizable solution. Once the desired drug-containing hydrophobic domains are obtained, they may be dispersed in a continuous polymerizable aqueous solution phase to form an emulsion. The emulsion is then polymerized to entrap and stabilize the hydrophobic microdomains. Oil soluble drugs may be dissolved in the hydrophobic phase while water soluble drugs may be micronized into this phase to form a fine dispersion.

15

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205).

- 20 Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., Pharmaceutical Research, 1994, 11, 1385; Ho et al., J. Pharm. Sci., 1996, 85, 138-143). Often  
25 microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. In one embodiment of this invention, the systems and methods of this invention which may incorporate microemulsions or components  
30 thereof, will facilitate the increased systemic absorption of compounds following crossing the skin barrier, as well as improve the local cellular uptake of the same within the cells comprising the respective skin layers.

Oil-in-water and water-in-oil microemulsions were prepared as exemplified herein, and comprised both a lipophilic and a hydrophilic compound. For oil-in-water microemulsions, increasing the hydrophilicity of the microemulsion resulted in greater retention versus permeation of the lipophilic or hydrophilic compound. For water-in-oil microemulsions, increasing the hydrophilicity of the microemulsion resulted in diminished retention and permeation of the lipophilic or hydrophilic compound.

Microemulsions comprising lipophilic or hydrophilic compounds were found to be efficiently taken up, retained, and passed through the skin. In one embodiment of this invention, the delivery systems of this invention, and methods of use thereof, may make use of microemulsions, for delivery of such compounds across the skin barrier.

Specific components of the microemulsions were evaluated similarly with lipophilic or hydrophilic compounds for their ability to be efficiently taken up, retained, and passed through the skin (Example 2). In each case, the compound delivered in a polar solvent, or a surfactant produced the greatest retention, while the organic solvent seemed to favor permeation, as did another surfactant, Tween 80. In each case, the crossing of the skin barrier was a function of the nature of the agent being delivered, the nature of the vehicle used, and in one embodiment, be a reflection of partitioning effects, which is also a function of the given chemistries. It will be apparent to one skilled in the art that based on the comparative studies presented here, general principles for drug delivery of various compounds across the skin barrier may be derived, and optimization accomplished, when the vehicle and agent chemistries are considered. Such optimization and considerations are to be considered as part of this invention, and an embodiment thereof, such that the components may be varied, and comprise any embodiment listed herein, and the agent may be any agent amenable to such manipulation.

In another embodiment of this invention, pretreatment of the skin enhances permeation and/or retention of a compound.

Pretreatment of the skin is accomplished with the use of a saturating agent, as described herein. In one embodiment, a saturating agent is a polar solvent, an organic solvent or a surfactant, and may comprise any embodiment herein for such materials.

In one embodiment, the saturating agent, which in use in formulating systems and for use in methods for promoting retention of a compound, will have a molecular weight of up to **1450 Da**.

In one embodiment, such pretreatment may comprise contacting skin with water, which may also be referred to herein as “hydration”, or, in another embodiment, may be referred to as “saturation”. In one embodiment, pretreatment may be for a period of time which facilitates greatest retention and/or permeation of the skin by a compound of this invention. Such a time may be determined experimentally, as exemplified herein, where optimal retention and/or permeation profiles are obtained, which correspond to a particular period of time of pretreatment. In one embodiment, pretreatment will be for a period of hours, prior to delivery of the compound in a respective vehicle, which in one embodiment, may range from 30 minutes – 18 hours, or in another embodiment, from 1-12 hours, or in another embodiment, from 12-18 hours. In one embodiment, skin exposure to the vehicle comprising the agent of this invention may also be for a period of time, which may range from 1-72 hours, the length of which may vary as a function of the physico-chemical properties of the agent, vehicle or saturating compound used, or application method. In one embodiment, such a time may be extended for a period of days, and reapplications may also be conducted, as will be appreciated by one skilled in the art, and will, in other embodiments, be varied to suit a particular application.

In one embodiment, the topical delivery systems of this invention for applications in skin retention of a lipophilic agent will comprise a saturating agent, which is a surfactant, or, in another embodiment, an organic solvent and in such systems, the vehicle may be water, or, in another embodiment, the vehicle may be a surfactant. In another embodiment, the saturating agent is a Span or a Tween and the vehicle is 1,2-octanediol, or in another embodiment, the saturating agent is 1,2-octanediol and the vehicle is a Span or Tween. In another embodiment, the saturating agent is an organic solvent and the vehicle is a surfactant, or in another embodiment, the saturating agent is a surfactant and the vehicle is an organic solvent.

In another embodiment, the topical delivery systems of this invention for lipophilic agent permeation of the skin, may comprise a saturating agent and a lipophilic agent in solution in an organic solvent or a surfactant. In one embodiment, the saturating agent is a polar solvent, an organic solvent or a surfactant, which, in another embodiment has a molecular weight of at least 18 Da. In another embodiment, the vehicle has a molecular weight of at least 18 Da.

In one embodiment, the saturating agent is a water or Tween and the vehicle is isopropyl myristate. In another embodiment, saturating agent is isopropyl myristate or Span and the vehicle is Tween. In another embodiment, the saturating agent is Tween and the vehicle is Span.

5 In another embodiment, this invention provides a topical delivery system, for retention of a hydrophilic agent, comprising a saturating agent and a hydrophilic drug in solution in a polar solvent, a surfactant or an organic solvent. In one embodiment, the saturating agent is a polar solvent, an organic solvent or a surfactant, and in another embodiment, has a molecular weight of up to 1450 Da. In another embodiment, the vehicle has a molecular weight of up to 1450 Da.

10 According to this aspect of the invention and in another embodiment, the saturating agent is an organic solvent, a surfactant, or a polar solvent, and the vehicle is water. In another embodiment, the saturating agent is an organic solvent and the vehicle is a surfactant. In another embodiment, the saturating agent is a surfactant and the vehicle is a surfactant. According to this aspect of the  
15 invention, and in one embodiment, the saturating agent is a Tween or 1, 2-octanediol and the vehicle is a Span. In another embodiment, the saturating agent is a Tween or a Span and the vehicle is 1, 2-octanediol. In another embodiment, the saturating agent is a surfactant and the vehicle is an organic solvent. In another embodiment, the saturating agent is an organic solvent and the vehicle is a surfactant.

20 Several factors determine the permeability of the skin to administered agents. These factors include the characteristics of the treated skin, the characteristics of the delivery agent, interactions between both the drug and delivery agent and the drug and skin, the dosage of the drug applied, the form of treatment, and the post treatment regimen. To selectively target the epidermis and dermis, it is  
25 sometimes possible to formulate a composition that comprises one or more penetration enhancers that will enable penetration of the drug to a preselected stratum.

In another embodiment, this invention provides a topical delivery system for hydrophilic agent permeation of the skin, comprising a saturating agent and a hydrophilic agent in solution in an  
30 organic solvent or a surfactant. In one embodiment, the saturating agent is a polar solvent, an organic solvent or a surfactant, which in another embodiment, has a molecular weight of at least 18 Da. In another embodiment, the vehicle has a molecular weight of at least 18 Da.

According to this aspect of the invention, and in one embodiment, the saturating agent is a water or Tween and the vehicle is isopropyl myristate. In another embodiment, the saturating agent is isopropyl myristate or Span and the vehicle is Tween. In another embodiment, the saturating agent is Tween and the vehicle is Span.

5

In one embodiment, formulation of a particular delivery system to promote retention within versus permeation of the skin is a relative determination, which, in one embodiment, may result in ratios (retention: permeation) of 1.5 – 400. In one embodiment, increasing the molecular weight of the vehicle used for delivery of the compound, or the saturating agent will influence the ratio, such as  
10 was exemplified herein, and representing one embodiment, when a hydrophilic molecule was delivered in an organic solvent, or, in another embodiment, when the hydrophilic molecule is delivered to skin saturated with an organic solvent (Example 5).

In another embodiment, this invention provides a method of transdermal drug delivery in a subject,  
15 comprising contacting a skin surface of said subject with a saturating agent and contacting the skin surface with a drug in a vehicle, wherein the vehicle comprises a polar solvent, a surfactant, an organic solvent or a combination thereof and whereby the drug is retained within, or permeates a skin in the subject, thereby being a method of transdermal drug delivery in the subject.

20 The systems and methods of this invention provide a means of a compound gaining access across the skin barrier, in one embodiment. The term “compound” and “drug” and “agent” are to be considered synonymous, when referred to herein, and represent a molecule whose access across the skin barrier is desired. In one embodiment, compounds for use in the systems and methods of this invention may comprise, *inter-alia*, an antibody or antibody fragment, a peptide, an oligonucleotide, a ligand for a  
25 biological target, an immunoconjugate, a chemomimetic functional group, a glycolipid, a labelling agent, an enzyme, a metal ion chelate, an enzyme cofactor, a cytotoxic compound, a bactericidal compound, a bacteriostatic compound, a chemotherapeutic, a growth factor, a hormone, a cytokine, a toxin, a prodrug, an antimetabolite, a microtubule inhibitor, a radioactive material, a targeting moiety, or any combination thereof.

30

In one embodiment, the term “antibody or antibody fragment” refers to intact antibody molecules as well as functional fragments thereof, such as Fab, F(ab')<sub>2</sub>, and Fv that are capable of binding to an epitope. In one embodiment, an Fab fragment refers to the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, which can be produced by digestion of whole

antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain. In one embodiment, Fab' fragment refers to a part of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain. Two Fab' fragments may be obtained per antibody molecule. In one  
5 embodiment, (Fab')<sub>2</sub> refers to a fragment of an antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction. In another embodiment, F(ab')<sub>2</sub> is a dimer of two Fab' fragments held together by two disulfide bonds. In one embodiment, Fv, may refer to a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains. In one embodiment, the antibody  
10 fragment may be a single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane,  
15 Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

In one embodiment, the antibody will recognize an epitope, which in another embodiment, refers to antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic  
20 determinants may, in other embodiments, consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and in other embodiments, may have specific three dimensional structural characteristics, and/or in other embodiments, have specific charge characteristics.

25 Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. In other embodiments, antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of  
30 antibodies with pepsin to provide a 5S fragment denoted F(ab')<sub>2</sub>. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos.



4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R., *Biochem. J.*, 73: 119-126, 1959. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al., *Proc. Nat'l Acad. Sci. USA* 69:2659-62, 1972. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow and Filpula, *Methods*, 2: 97-105, 1991; Bird et al., *Science* 242:423-426, 1988; Pack et al., *Bio/Technology* 11:1271-77, 1993; and Ladner et al., U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry, *Methods*, 2: 106-10, 1991.

In one embodiment, the term "peptide" refers to native peptides (either degradation products, synthetically synthesized peptides or recombinant peptides) and/or peptidomimetics (typically, synthetically synthesized peptides), such as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the peptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to N terminus modification, C terminus modification, peptide bond modification, including, but not limited to,  $\text{CH}_2\text{-NH}$ ,  $\text{CH}_2\text{-S}$ ,  $\text{CH}_2\text{-S=O}$ ,  $\text{O=C-NH}$ ,  $\text{CH}_2\text{-O}$ ,  $\text{CH}_2\text{-CH}_2$ ,  $\text{S=C-NH}$ ,  $\text{CH=CH}$  or  $\text{CF=CH}$ , backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in *Quantitative Drug Design*, C.A. Ramsden Gd.,

Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinunder.

Peptide bonds (-CO-NH-) within the peptide may be substituted, for example, by N-methylated  
5 bonds (-N(CH<sub>3</sub>)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-CH<sub>2</sub>-), aza  
bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH<sub>2</sub>-NH-),  
hydroxyethylene bonds (-CH(OH)-CH<sub>2</sub>-), thioamide bonds (-CS-NH-), olefinic double bonds (-  
CH=CH-), retro amide bonds (-NH-CO-), peptide derivatives (-N(R)-CH<sub>2</sub>-CO-), wherein R is the  
"normal" side chain, naturally presented on the carbon atom. These modifications can occur at any  
10 of the bonds along the peptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid  
such as TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of  
Phe or o-methyl-Tyr. In addition to the above, the peptides of the present invention may also include  
15 one or more modified amino acids or one or more non-amino acid monomers (e.g. fatty acids,  
complex carbohydrates etc).

In one embodiment, the term "amino acid" or "amino acids" is understood to include the 20 naturally  
occurring amino acids; those amino acids often modified post-translationally in vivo, including, for  
20 example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids  
including, but not limited to, 2-amino adipic acid, hydroxylysine, isodesmosine, nor-valine, nor-  
leucine and ornithine. Furthermore, the term "amino acid" may include both D- and L-amino acids.

In one embodiment, the term "oligonucleotide" is interchangeable with the term "nucleic acid", and  
25 may refer to a molecule, which may include, but is not limited to, prokaryotic sequences, eukaryotic  
mRNA, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g.,  
mammalian) DNA, and even synthetic DNA sequences. The term also refers to sequences that  
include any of the known base analogs of DNA and RNA.

30 The systems of this invention may comprise nucleic acids, in one embodiment, or in another  
embodiment, the methods of this invention may include delivery of the same, wherein, in another  
embodiment, the nucleic acid is a part of a particular vector. In one embodiment, polynucleotide  
segments encoding sequences of interest can be ligated into commercially available expression  
vector systems suitable for transducing/transforming mammalian cells and for directing the

expression of recombinant products within the transduced cells. It will be appreciated that such commercially available vector systems can easily be modified via commonly used recombinant techniques in order to replace, duplicate or mutate existing promoter or enhancer sequences and/or introduce any additional polynucleotide sequences such as for example, sequences encoding  
5 additional selection markers or sequences encoding reporter polypeptides .

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (e.g., Southern blotting) and RNA  
10 produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity of the gene product, such as an enzymatic assay. If the gene product of interest to be expressed by a cell is  
15 not readily assayable, an expression system can first be optimized using a reporter gene linked to the regulatory elements and vector to be used. The reporter gene encodes a gene product, which is easily detectable and, thus, can be used to evaluate efficacy of the system. Standard reporter genes used in the art include genes encoding  $\beta$ -galactosidase, chloramphenicol acetyl transferase, luciferase and human growth hormone.

As will be appreciated by one skilled in the art, a fragment or derivative of a nucleic acid sequence or  
20 gene that encodes for a protein or peptide can still function in the same manner as the entire, wild type gene or sequence. Likewise, forms of nucleic acid sequences can have variations as compared to wild type sequences, nevertheless encoding the protein or peptide of interest, or fragments thereof, retaining wild type function exhibiting the same biological effect, despite these variations. Each of  
25 these represents a separate embodiment of this present invention.

The nucleic acids can be produced by any synthetic or recombinant process such as is well known in the art. Nucleic acids can further be modified to alter biophysical or biological properties by means of techniques known in the art. For example, the nucleic acid can be modified to increase its stability  
30 against nucleases (e.g., "end-capping"), or to modify its solubility, or binding affinity to complementary sequences. In another embodiment, its lipophilicity may be modified, which, in turn, will reflect changes in the systems employed for its delivery, and in one embodiment, may further be influenced by whether such sequences are desired for retention within, or permeation through the

skin, or any of its layers. Such considerations may influence any compound used in this invention, in the methods and systems described.

Methods for modifying nucleic acids to achieve specific purposes are disclosed in the art, for example, in Sambrook et al. (1989). Moreover, the nucleic acid sequences of the invention can include one or more portions of nucleotide sequence that are non-coding for the protein of interest. Variations in the DNA sequences, which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the polypeptides encoded thereby, are also encompassed in the invention.

In one embodiment, the agent is one which may inhibit gene expression in a subject. In one embodiment, the agent that inhibits gene expression, activity or function comprises a nucleic acid. The nucleic acid may, in one embodiment, be DNA, or in another embodiment, the nucleic acid is RNA. In other embodiments, the nucleic acid may be single or double stranded. In another embodiment, the agent is a nucleic acid that is antisense in orientation to a sequence encoding for a caspase.

In one embodiment, the agents used in the systems and methods of this invention may be used for gene silencing applications. In one embodiment, the activity or function of a particular gene is suppressed or diminished, via the use of antisense oligonucleotides, which are chimeric molecules, containing two or more chemically distinct regions, each made up of at least one nucleotide. In one embodiment, the antisense molecules may be conjugated to the polymers of this invention, as described, or in another embodiment, encapsulated within micelles of this invention, much as any of the respective groups listed herein, applicable in the methods of this invention, in another embodiment, may be conjugated to the polymers of this invention, or encapsulated within micelles of this invention.

Antisense oligonucleotides, in one embodiment, may be chimeric oligonucleotides, which contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide an increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target polynucleotide. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids, which according to this aspect of the invention, serves as a means of gene silencing via degradation of specific sequences.

Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

The chimeric antisense oligonucleotides may, in one embodiment, be formed as composite structures of two or more oligonucleotides and/or modified oligonucleotides, as is well described in the art (see, for example, U.S. Pat. Nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922), and can, in another embodiment, comprise a ribozyme sequence.

Inhibition of gene expression, activity or function is effected, in another embodiment, via the use of small interfering RNAs, which provides sequence-specific inhibition of gene expression. Administration of double stranded/duplex RNA (dsRNA) corresponding to a single gene in an organism can silence expression of the specific gene by rapid degradation of the mRNA in affected cells. This process is referred to as gene silencing, with the dsRNA functioning as a specific RNA inhibitor (RNAi). RNAi may be derived from natural sources, such as in endogenous virus and transposon activity, or it can be artificially introduced into cells (Elbashir SM, et al (2001). Nature 411:494-498) via microinjection (Fire et al. (1998) Nature 391: 806-11), or by transformation with gene constructs generating complementary RNAs or fold-back RNA, or by other vectors (Waterhouse, P.M., et al. (1998). Proc. Natl. Acad. Sci. USA 95, 13959-13964 and Wang, Z., et al. (2000). J. Biol. Chem. 275, 40174-40179). The RNAi mediating mRNA degradation, in one embodiment, comprises duplex or double-stranded RNA, or, in other embodiments, include single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion and/or alteration of one or more nucleotides.

In another embodiment, gene expression can be inhibited/downregulated simply by “knocking out” the gene. Typically this is accomplished by disrupting the gene, the promoter regulating the gene or sequences between the promoter and the gene. Such disruption can be specifically directed to a particular gene by homologous recombination where a “knockout construct” contains flanking sequences complementary to the domain to which the construct is targeted. Insertion of the knockout construct (e.g. into the gene of interest) results in disruption of that gene. The phrases “disruption of the gene” and “gene disruption” refer to insertion of a nucleic acid sequence into one region of the native DNA sequence (in some embodiments, in one or more exons) and/or the promoter region of a

gene so as to decrease or prevent expression of that gene in the cell as compared to the wild-type or naturally occurring sequence of the gene.

Knockout constructs can be produced by standard methods known to those of skill in the art. The knockout construct can be chemically synthesized or assembled, e.g., using recombinant DNA methods. The DNA sequence to be used in producing the knockout construct is digested with a particular restriction enzyme selected to cut at a location(s) such that a new DNA sequence encoding a marker gene can be inserted in the proper position within this DNA sequence. The proper position for marker gene insertion is that which will serve to prevent expression of the native gene; this position will depend on various factors such as the restriction sites in the sequence to be cut, and whether an exon sequence or a promoter sequence, or both is (are) to be interrupted (i.e., the precise location of insertion necessary to inhibit promoter function or to inhibit synthesis of the native exon).

It is to be understood that the above nucleic acids may be delivered to any tissue or cells in one embodiment, in their native form, or, in another embodiment within an expression vector that is competent to transfect cells in vitro and/or in vivo, and comprise an embodiment of this invention.

In one embodiment, the nucleic acid encodes for an antibacterial, antiviral, antifungal or antiparasitic peptide or protein. In another embodiment, the nucleic acid encodes for a peptide or protein with cytotoxic or anti-cancer activity. In another embodiment, the nucleic acid encodes for an enzyme, a receptor, a channel protein, a hormone, a cytokine or a growth factor. In another embodiment, the nucleic acid encodes for a peptide or protein, which is immunostimulatory. In another embodiment, the nucleic acid encodes for a peptide or protein, which inhibits inflammatory or immune responses. In another embodiment, release of the nucleic acid occurs over a period of time.

In one embodiment, the systems of this invention are targeted to specific cells. In one embodiment, the cell may be any responsive cell, such as, in one embodiment, an epithelial cell, a lung cell, a kidney cell, a liver cell, a cardiocyte, an astrocyte, a glial cell, a prostate cell, a professional antigen presenting cell, a lymphocyte, an M cell, a pancreatic cell, a stem cell, a myoblast, a hepatocyte, an osteoblast, an osteocyte, an osteoclast, a chondrocyte, a chondroblast, or other bone or cartilage cells and may be used for applications as described in, for example, Wilson, J. M et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano, D. et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Wolff, J. A. et al. (1990) Science 247:1465-1468; Chowdhury, J. R. et al. (1991) Science 254:1802-1805; Ferry, N. et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Wilson, J. M. et

al. (1992) J. Biol. Chem. 267:963-967; Quantin, B. et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584; Dai, Y. et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; van Beusechem, V. W. et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Rosenfeld, M. A. et al. (1992) Cell 68:143-155; Kay, M. A. et al. (1992) Human Gene Therapy 3:641-647; Cristiano, R. J. et al. (1993) Proc. Natl. Acad. Sci. USA 90:2122-2126; Hwu, P. et al. (1993) J. Immunol. 150:4104-4115; and Herz, J. and Gerard, R. D. (1993) Proc. Natl. Acad. Sci. USA 90:2812-2816.

Targeting may be accomplished, in one embodiment, via the incorporation of a targeting moiety in the external phase of a microemulsion, as described herein, or in another embodiment, via conjugation of the targeting moiety to an agent whose targeted delivery is desired.

In one embodiment, the "drug" or "compound" or "agent" for use in the systems and methods of this invention, is a substance applicable for use in the diagnosis, or in another embodiment, cure, or in another embodiment, mitigation, or in another embodiment, treatment, or in another embodiment, prevention of a disease, disorder, condition or infection. In one embodiment, the "drug" or "compound" or "agent" for use in the systems and methods of this invention, refers to any substance which affect the structure or function of the target to which it is applied.

In another embodiment, the "drug" or "compound" or "agent" for use in the systems and methods of this invention, is a molecule that alleviates a symptom of a disease or disorder when administered to a subject afflicted thereof. In one embodiment, the "drug" or "compound" or "agent" for use in the systems and methods of this invention is a synthetic molecule, or in another embodiment, a naturally occurring compound isolated from a source found in nature.

In one embodiment, the "drug" or "compound" or "agent" for use in the systems and methods of this invention may comprise antihypertensives, antidepressants, antianxiety agents, anticlotting agents, anticonvulsants, blood glucose-lowering agents, decongestants, antihistamines, antitussives, anti-inflammatories, antipsychotic agents, cognitive enhancers, cholesterol-reducing agents, antiobesity agents, autoimmune disorder agents, anti-impotence agents, antibacterial and antifungal agents, hypnotic agents, anti-Parkinsonism in agents, antibiotics, antiviral agents, anti-neoplastics, barbituates, sedatives, nutritional agents, beta blockers, emetics, anti-emetics, diuretics, anticoagulants, cardiotonics, androgens, corticoids, anabolic agents, growth hormone secretagogues, anti-infective agents, coronary vasodilators, carbonic anhydrase inhibitors, antiprotozoals, gastrointestinal agents, serotonin antagonists, anesthetics, hypoglycemic agents, dopaminergic

agents, anti-Alzheimer's Disease agents, anti-ulcer agents, platelet inhibitors and glycogen phosphorylase inhibitors.

In one embodiment, examples of the "drug" or "compound" or "agent" for use in the systems and methods of this invention comprise, *inter-alia*, antihypertensives including prazosin, nifedipine, trimazosin, amlodipine, and doxazosin mesylate; the antianxiety agent hydroxyzine; a blood glucose lowering agent such as glipizide; an anti-impotence agent such as sildenafil citrate; anti-neoplastics such as chlorambucil, lomustine or echinomycin; anti-inflammatory agents such as betamethasone, prednisolone, piroxicam, aspirin, flurbiprofen and (+)-N-{4-[3-(4-fluorophenoxy)phenoxy]-2-cyclopenten-1-yl}-N-hydroxyurea; antivirals such as acyclovir, nelfinavir, or virazole; vitamins/nutritional agents such as retinol and vitamin E; emetics such as apomorphine; diuretics such as chlorthalidone and spironolactone; an anticoagulant such as dicumarol; cardiotonics such as digoxin and digitoxin; androgens such as 17-methyltestosterone and testosterone; a mineral corticoid such as desoxycorticosterone; a steroidal hypnotic/anesthetic such as alfaxalone; an anabolic agent such as fluoxymesterone or methanstenolone; antidepressants such as fluoxetine, pyroxidine, venlafaxine, sertraline, paroxetine, sulpiride, [3,6-dimethyl-2-(2,4,6-trimethyl-phenoxy)-pyridin-4-yl]-(1-ethylpropyl)-amine or 3,5-dimethyl-4-(3'-pentoxy)-2-(2',4',6'-trimethylphenoxy)pyridine; an antibiotic such as ampicillin and penicillin G; an anti-infective such as benzalkonium chloride or chlorhexidine; a coronary vasodilator such as nitroglycerin or miflozine; a hypnotic such as etomidate; a carbonic anhydrase inhibitor such as acetazolamide or chlorzamide; an antifungal such as econazole, terconazole, fluconazole, voriconazole or griseofulvin; an antiprotozoal such as metronidazole; an imidazole-type anti-neoplastic such as tubulazole; an anthelmintic agent such as thiabendazole or oxfendazole; an antihistamine such as astemizole, levocabastine, cetirizine, or cinnarizine; a decongestant such as pseudoephedrine; antipsychotics such as fluspirilene, penfluridole, risperidone or ziprasidone; a gastrointestinal agent such as loperamide or cisapride; a serotonin antagonist such as ketanserin or mianserin; an anesthetic such as lidocaine; a hypoglycemic agent such as acetohexamide; an anti-emetic such as dimenhydrinate; an antibacterial such as cotrimoxazole; a dopaminergic agent such as L-DOPA; anti-Alzheimer agents such as THA or donepezil; an anti-ulcer agent/H<sub>2</sub> antagonist such as famotidine; a sedative/hypnotic such as chlordiazepoxide or triazolam; a vasodilator such as alprostadil; a platelet inhibitor such as prostacyclin; an ACE inhibitor/antihypertensive such as enalaprilic acid or lisinopril; a tetracycline antibiotic such as oxytetracycline or minocycline; a macrolide antibiotic such as azithromycin, clarithromycin, erythromycin or spiramycin; and glycogen phosphorylase inhibitors such as [R-(R\*S\*)]-5-chloro-N-[2-hydroxy-3{methoxymethylamino}-3-oxo-1-(phenylmethyl)-propyl]-IH-



indole-2-carboxamide or 5-chloro-1 -Hindole-2-carboxylic acid [(IS)-benzyl(2R)-hydroxy-3-((3R,4S)dihydroxy-pyrrolidin-1-yl)-oxypropyl] amide.

Further examples of the “drug” or “compound” or “agent” for use in the systems and methods of this invention are the glucose-lowering drug chlorpropamide, the anti-fungal fluconazole, the anti-hypercholesterolemic atorvastatin calcium, the antipsychotic thiothixene hydrochloride, the anxiolytics hydroxyzine hydrochloride or doxepin hydrochloride, the anti-hypertensive amlodipine besylate, the antiinflammatories piroxicam and celicoxib and valdicoxib, and the antibiotics carbenicillin indanyl sodium, bacampicillin hydrochloride, troleandomycin, and doxycycline hyclate.

In another embodiment a “drug” or “compound” or “agent” for use in the systems and methods of this invention may comprise other antineoplastic agents such as platinum compounds (e.g., spiroplatin, cisplatin, and carboplatin), methotrexate, fluorouracil, adriamycin, mitomycin, ansamitocin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopolylysine, vincristine, busulfan, chlorambucil, melphalan (e.g., PAM, L-PAM or phenylalanine mustard), mercaptopurine, mitotane, procarbazine hydrochloride dactinomycin (actinomycin D), daunorubicin hydrochloride, doxorubicin hydrochloride, paclitaxel and other taxenes, rapamycin, manumycin A, TNP-470, plicamycin (mithramycin), aminoglutethimide, estramustine phosphate sodium, flutamide, leuprolide acetate, megestrol acetate, tamoxifen citrate, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase) Erwinia asparaginase, interferon .alpha.-2a, interferon .alpha.-2b, teniposide (VM-26), vinblastine sulfate (VLB), vincristine sulfate, bleomycin sulfate, hydroxyurea, procarbazine, and dacarbazine; mitotic inhibitors such as etoposide, colchicine, and the vinca alkaloids, radiopharmaceuticals such as radioactive iodine and phosphorus products; hormones such as progestins, estrogens and antiestrogens; anti-helminthics, antimalarials, and antituberculosis drugs; biologicals such as immune serums, antitoxins and antivenoms; rabies prophylaxis products; bacterial vaccines; viral vaccines; respiratory products such as xanthine derivatives theophylline and aminophylline; thyroid agents such as iodine products and anti-thyroid agents; cardiovascular products including chelating agents and mercurial diuretics and cardiac glycosides; glucagon; blood products such as parenteral iron, hemin, hematoporphyrins and their derivatives; biological response modifiers such as muramyl dipeptide, muramyl tripeptide, microbial cell wall components, lymphokines (e.g., bacterial endotoxin such as lipopolysaccharide, macrophage activation factor), sub-units of bacteria (such as Mycobacteria, Corynebacteria), the synthetic dipeptide N-acetyl-muramyl-L-alanyl-D-isoglutamine; anti-fungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine (5-fc), miconazole, amphotericin B, ricin, cyclosporins, and  $\beta$ -lactam antibiotics (e.g.,

sulfazecin); hormones such as growth hormone, melanocyte stimulating hormone, estradiol, beclomethasone dipropionate, betamethasone, betamethasone acetate and betamethasone sodium phosphate, betamethasone disodium phosphate, betamethasone sodium phosphate, cortisone acetate, dexamethasone, dexamethasone acetate, dexamethasone sodium phosphate, flunisolide, hydrocortisone, hydrocortisone acetate, hydrocortisone cypionate, hydrocortisone sodium phosphate, hydrocortisone sodium succinate, methylprednisolone, methylprednisolone acetate, methylprednisolone sodium succinate, paramethasone acetate, prednisolone, prednisolone acetate, prednisolone sodium phosphate, prednisolone tebutate, prednisone, triamcinolone, triamcinolone acetonide, triamcinolone diacetate, triamcinolone hexacetonide, fludrocortisone acetate, oxytocin, vasopressin, and their derivatives; vitamins such as cyanocobalamin, folic acid, retinoids and derivatives such as retinol palmitate, and  $\alpha$ -tocopherol; peptides, such as manganese superoxide dismutase; enzymes such as alkaline phosphatase; anti-allergic agents such as amlexanox; anti-coagulation agents such as phenprocoumon and heparin; circulatory drugs such as propranolol; metabolic potentiators such as glutathione; antituberculars such as para-aminosalicylic acid, isoniazid, capreomycin sulfate, cycloserine, ethambutol hydrochloride, ethionamide, pyrazinamide, rifampin, and streptomycin sulfate; antivirals such as amantadine, azidothymidine (AZT, DDI, Foscarnet, or Zidovudine), ribavirin and vidarabine monohydrate (adenine arabinoside, ara-A); antianginals such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide dinitrate, nitroglycerin (glyceryl trinitrate) and pentaerythritol tetranitrate; anticoagulants such as phenprocoumon, heparin; antibiotics such as dapson, chloramphenicol, neomycin, cefaclor, cefadroxil, cephalixin, cephadrine, erythromycin, clindamycin, lincomycin, amoxicillin, ampicillin, bacampicillin, carbenicillin, dicloxacillin, cyclacillin, picloxacillin, hetacillin, methicillin, nafcillin, oxacillin, penicillin including penicillin G and penicillin V, ticarcillin, rifampin and tetracycline; antiinflammatories such as diflunisal, ibuprofen, indomethacin, meclofenamate, mefenamic acid, naproxen, oxyphenbutazone, phenylbutazone, piroxicam, sulindac, tolmetin, aspirin and salicylates; antiprotozoans such as chloroquine, hydroxychloroquine, metronidazole, quinine and meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric; opiates such as codeine, heroin, methadone, morphine and opium; cardiac glycosides such as deslanoside, digitoxin, digoxin, digitalin and digitalis; neuromuscular blockers such as atracurium mesylate, gallamine triethiodide, hexafluorenum bromide, metocurine iodide, pancuronium bromide, succinylcholine chloride (suxamethonium chloride), tubocurarine chloride and vecuronium bromide; sedatives (hypnotics) such as amobarbital, amobarbital sodium, aprobarbital, butobarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotrimeprazine hydrochloride, methypylon, midazolam hydrochloride, paraldehyde, pentobarbital, pentobarbital

sodium, phenobarbital sodium, secobarbital sodium, talbutal, temazepam and triazolam; local anesthetics such as bupivacaine hydrochloride, chloroprocaine hydrochloride, etidocaine hydrochloride, lidocaine hydrochloride, mepivacaine hydrochloride, procaine hydrochloride and tetracaine hydrochloride; general anesthetics such as droperidol, etomidate, fentanyl citrate with droperidol, ketamine hydrochloride, methohexital sodium and thiopental sodium; and radioactive particles or ions such as strontium, iodide rhenium and yttrium.

In one embodiment, the “drug” or “compound” or “agent” for use in the systems and methods of this invention is a therapeutic compound. In one embodiment, the therapeutic compound is a peptide, a protein or a nucleic acid. In another embodiment, the therapeutic compound is an antibacterial, antiviral, antifungal or antiparasitic compound. In another embodiment, the therapeutic compound has cytotoxic or anti-cancer activity. In another embodiment, the therapeutic compound is an enzyme, a receptor, a channel protein, a hormone, a cytokine or a growth factor. In another embodiment, the therapeutic compound is immunostimulatory. In another embodiment, the therapeutic compound inhibits inflammatory or immune responses.

In one embodiment, the term “therapeutic”, refers to a molecule, which when provided to a subject in need, provides a beneficial effect. In some cases, the molecule is therapeutic in that it functions to replace an absence or diminished presence of such a molecule in a subject. In one embodiment, the molecule is a nucleic acid coding for the expression of a protein is absent, such as in cases of an endogenous null mutant being compensated for by expression of the foreign protein. In other embodiments, the endogenous protein is mutated, and produces a non-functional protein, compensated for by the expression of a heterologous functional protein. In other embodiments, expression of a heterologous protein is additive to low endogenous levels, resulting in cumulative enhanced expression of a given protein. In other embodiments, the molecule stimulates a signalling cascade that provides for expression, or secretion, or others of a critical element for cellular or host functioning. In one embodiment, the therapeutic compound is a protein or polypeptide.

In one embodiment, the therapeutic protein may include cytokines, such as interferons or interleukins, or their receptors. Lack of expression of cytokines, or of the appropriate ones, has been implicated in susceptibility to diseases, and enhanced expression may lead to resistance to a number of infections. Expression patterns of cytokines may be altered to produce a beneficial effect, such as for example, a biasing of the immune response toward a Th1 type expression pattern, or a Th2

pattern in infection, or in autoimmune disease, wherein altered expression patterns may prove beneficial to the host.

In another embodiment, the therapeutic protein may comprise an enzyme, such as one involved in glycogen storage or breakdown. In another embodiment, the therapeutic protein comprises a transporter, such as an ion transporter, for example CFTR, or a glucose transporter, or other transporters whose deficiency, or inappropriate expression, results in a variety of diseases.

In another embodiment, the therapeutic protein comprises a tumor suppressor, or pro-apoptotic compound, which alters progression of cancer-related events.

In another embodiment, the therapeutic compound of the present invention may comprise an immunomodulating protein. In one embodiment, the immunomodulating protein comprises cytokines, chemokines, complement or components, such as interleukins 1 to 15, interferons alpha, beta or gamma, tumour necrosis factor, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines such as neutrophil activating protein (NAP), macrophage chemoattractant and activating factor (MCAF), RANTES, macrophage inflammatory peptides MIP-1a and MIP-1b, or complement components.

In another embodiment, the therapeutic molecule may be natural or non-natural insulins, amylases, proteases, lipases, kinases, phosphatases, glycosyl transferases, trypsinogen, chymotrypsinogen, carboxypeptidases, hormones, ribonucleases, deoxyribonucleases, triacylglycerol lipase, phospholipase A2, elastases, amylases, blood clotting factors, UDP glucuronyl transferases, ornithine transcarbamoylases, cytochrome p450 enzymes, adenosine deaminases, serum thymic factors, thymic humoral factors, thymopoietins, growth hormones, somatomedins, costimulatory factors, antibodies, colony stimulating factors, erythropoietin, epidermal growth factors, hepatic erythropoietic factors (hepatopoietin), liver-cell growth factors, interleukins, interferons, negative growth factors, fibroblast growth factors, transforming growth factors of the  $\alpha$  family, transforming growth factors of the  $\beta$  family, gastrins, secretins, cholecystokinins, somatostatins, serotonin, substance P, transcription factors or combinations thereof.

In one embodiment, the systems of this invention, and materials for use in the methods of this invention may further comprise a ligand for a biological target, which in another embodiment,

provides for directional specificity as to which cells or tissues are targeted, as described hereinabove. In one embodiment, the term "ligand for a biological target" refers to a molecule which enables the specific delivery of the compounds/systems of this invention to a particular site *in vivo*. In one embodiment, such a ligand may be referred to as an "anti-receptor", which functions to direct the compounds/systems of this invention to, for example, virally infected cells, via anti-receptor binding to viral proteins expressed on infected cell surfaces. In this case, antireceptors to promote fusion with virally-infected cells, will recognize and bind to virally expressed surface proteins. For example, HIV-1 infected cells may express HIV-associated proteins, such as gp120, and therefore the presence of CD4 on the polymer or micelle surface promotes targeting to HIV infected cells, via CD4-gp120 interaction.

In another embodiment, the systems of this invention, and materials in use for the methods of this invention, may comprise vaccines. In one embodiment, the term "vaccine" include, inter-alia, a peptide, or peptides, which induce specific immune responses to an organism from which the peptide/s were isolated, or modeled after. Such peptide/s may be derived from a pathogen, or a pathogenic cell.

Bacterial proteins expressed during intracellular infection are such potential targets contemplated for therapeutic intervention by the systems/materials for use according to this invention. The intracellular bacteria may include, amongst others: Shigella, Salmonella, Legionella, Streptococci, Mycobacteria, Francisella and Chlamydiae (See G. L. Mandell, "Introduction to Bacterial Disease" IN CECIL TEXTBOOK OF MEDICINE, (W.B. Saunders Co., 1996) 1556-7). These bacteria would be expected to express a bacteria-related protein on the surface of the infected cell to which the polymers/micelles of this invention would attach.

Proteins of parasitic agents, which reside intracellularly, may similarly be applied. The intracellular parasites contemplated include for example, Protozoa. Protozoa, which infect cells, include: parasites of the genus Plasmodium (e.g., Plasmodium falciparum, P. Vivax, P. ovale and P. malariae), Trypanosoma, Toxoplasma, Leishmania, and Cryptosporidium.

Diseased and/or abnormal cells may be treated using the systems of this invention, and according to the methods of this invention. The diseased or abnormal cells contemplated include: infected cells, neoplastic cells, pre-neoplastic cells, inflammatory foci, benign tumors or polyps, cafe au lait spots, leukoplakia, other skin moles, self-reactive cells, including T and/or NK cells, etc. Any cell, to

which delivery of an agent is desired, and is accomplished via using the systems or according to the methods of this invention, represents an embodiment of this invention.

In one embodiment, a therapeutic agent, or in another embodiment, a cytotoxic agent is delivered with the systems of this invention, and/or according to the methods of this invention. In some embodiments, the agent specifically acts on only diseased cells. In one embodiment, the agent is an antibody, or fragment thereof. Examples of antibodies include those antibodies, which react with malignant prostatic epithelium but not with benign prostate tissue (e.g., ATCC No. HB-9119; ATCC HB-9120; and ATCC No. HB-1 1430) or react with malignant breast cancer cells but not with normal breast tissue (e.g., ATCC No. HB-8691; ATCC No. HB-10807; and 21HB-108011). Other antibodies or fragments thereof, which react with diseased tissue and not with normal tissue, would be apparent to the skilled artisan.

A wide variety of tumor-specific antibodies are known in the art, such as those described in U.S. Pat. Nos. 6,197,524, 6,191,255, 6,183,971, 6,162,606, 6,160,099, 6,143,873, 6,140,470, 6,139,869, 6,113,897, 6,106,833, 6,042,829, 6,042,828, 6,024,955, 6,020,153, 6,015,680, 5,990,297, 5,990,287, 5,972,628, 5,972,628, 5,959,084, 5,951,985, 5,939,532, 5,939,532, 5,939,277, 5,885,830, 5,874,255, 5,843,708, 5,837,845, 5,830,470, 5,792,616, 5,767,246, 5,747,048, 5,705,341, 5,690,935, 5,688,657, 5,688,505, 5,665,854, 5,656,444, 5,650,300, 5,643,740, 5,635,600, 5,589,573, 5,576,182, 5,552,526, 5,532,159, 5,525,337, 5,521,528, 5,519,120, 5,495,002, 5,474,755, 5,459,043, 5,427,917, 5,348,880, 5,344,919, 5,338,832, 5,298,393, 5,331,093, 5,244,801, and 5,169,774. See also The Monoclonal Antibody Index Volume 1: Cancer (3rd edition). Accordingly, the delivery systems of this invention may comprise tumor-specific antibodies which may recognize tumors derived from a wide variety of tissue types, including, but not limited to, breast, prostate, colon, lung, pharynx, thyroid, lymphoid, lymphatic, larynx, esophagus, oral mucosa, bladder, stomach, intestine, liver, pancreas, ovary, uterus, cervix, testes, dermis, bone, blood and brain.

In another embodiment, the delivery systems of this invention will incorporate an antibody which possesses tumoricidal activity. Antibodies that possess tumoricidal activity are also known in the art, including IMC-C225, EMD 72000, OvaRex Mab B43.13, anti-ganglioside G(D2) antibody ch14.18, CO17-1A, trastuzumab, rhuMab VEGF, sc-321, AF349, BAF349, AF743, BAF743, MAB743, AB1875, Anti-Flt-4AB3127, FLT41-A, rituximab, 2C3, CAMPATH 1H, 2G7, Alpha IR-3, ABX-EGF, MDX-447, anti-p75 IL-2R, anti-p64 IL-2R, and 2A11.

Epitopes to which tumor-specific antibodies bind are also well known in the art. For example, epitopes bound by the tumor-specific antibodies of the invention include, but are not limited to, those known in the art to be present on CA-125, gangliosides G(D2), G(M2) and G(D3), CD20, CD52, CD33, Ep-CAM, CEA, bombesin-like peptides, PSA, HER2/neu, epidermal growth factor receptor, erbB2, erbB3, erbB4, CD44v6, Ki-67, cancer-associated mucin, VEGF, VEGFRs (e.g., VEGFR3), estrogen receptors, Lewis-Y antigen, TGF $\beta$ 1, IGF-1 receptor, EGF $\alpha$ , c-Kit receptor, transferrin receptor, IL-2R and CO17-1A. It is to be understood that antibodies to these, and other epitopes, may be designed by methods well known in the art, such as, for example, as described in Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (Harlow and Lane, 1988), or "Current Protocols in Immunology" (Coligan, 1991), and may be incorporated in the delivery systems of this invention, and represents embodiments thereof.

In one embodiment, the delivery systems of this invention may comprise a toxin. In one embodiment, the term "toxin" refers to a molecule which results in toxic effects in cells and/or tissue exposed to the toxin. In one embodiment, the toxin results in cell death, or in another embodiment, cell damage. In one embodiment, the toxin is a natural product of cells, such as bacterial cells, wherein the toxin is used, in one embodiment, when specifically targeted to disease cells as a means of selective cell killing of diseased cells. In one embodiment, the toxin may comprise any known in the art, such as, for example that produced by cholera, tetanus, or any other appropriate species, as will be appreciated by one skilled in the art.

In another embodiment, this invention also comprises incorporation of any toxic substance for therapeutic purpose. In one embodiment, the delivery systems of this invention may incorporate an oligonucleotide encoding a suicide gene, which when in contact with diseased cells or tissue, is expressed within such cells. In one embodiment, the term "suicide gene" refers to a nucleic acid coding for a product, wherein the product causes cell death by itself or in the presence of other compounds. A representative example of a suicide gene is one, which codes for thymidine kinase of herpes simplex virus. Additional examples are thymidine kinase of varicella zoster virus and the bacterial gene cytosine deaminase, which can convert 5-fluorocytosine to the highly cytotoxic compound 5-fluorouracil.

Suicide genes may produce cytotoxicity by converting a prodrug to a product that is cytotoxic. In one embodiment, the term "prodrug" means any compound that can be converted to a toxic product for cells. Representative examples of such a prodrug is gancyclovir which is converted in vivo to a

toxic compound by HSV-thymidine kinase. The gancyclovir derivative subsequently is toxic to cells. Other representative examples of prodrugs include acyclovir, FIAU [1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil], 6-methoxypurine arabinoside for VZV-TK, and 5-fluorocytosine for cytosine deaminase.

5

In one embodiment, the delivery systems of this invention and methods of this invention are for prevention of, or therapeutic intervention of viral infection. Examples of infectious virus to which such materials and methods are directed include: Retroviridae (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (e.g., polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g., strains that cause gastroenteritis); Togaviridae (e.g., equine encephalitis viruses, rubella viruses); Flaviridae (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g., coronaviruses); Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g., ebola viruses); Paramyxoviridae (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bungaviridae (e.g., Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses); Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g., the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

25

In one embodiment, the delivery systems of this invention and methods of this invention are for prevention of, or therapeutic intervention of bacterial infection. Examples of bacteria to which such materials and methods are directed include: *Helicobacter pylori*, *Borellia burgdorferi*, *Legionella pneumophila*, *Mycobacteria* sps (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter* sp.,

30



*Enterococcus* sp., *Chlamidia* sp., *Haemophilus influenzae*, *Bacillus anthracis*, *corynebacterium diphtheriae*, *corynebacterium* sp., *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides* sp., *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*,  
5 *Treponema pertenue*, *Leptospira*, *Actinomyces israeli* and *Francisella tularensis*.

In one embodiment, the delivery systems of this invention and methods of this invention are for prevention of, or therapeutic intervention of fungal infection. Examples of fungi to which such materials and methods are directed include: *Cryptococcus neoformans*, *Histoplasma capsulatum*,  
10 *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*. Other infectious organisms (i.e., protists) to which the delivery systems of this invention and methods of this invention are directed may include: *Plasmodium* sp., *Leishmania* sp., *Schistosoma* sp. and *Toxoplasma* sp.

15 According to this aspect of the invention, the disease for which the subject is thus treated may comprise, but is not limited to: muscular dystrophy, cancer, cardiovascular disease, hypertension, infection, renal disease, neurodegenerative disease, such as alzheimer's disease, parkinson's disease, huntington's chorea, Creutzfeld-Jacob disease, autoimmune disease, such as lupus, rheumatoid arthritis, endocarditis, Graves' disease or ALD, respiratory disease such as asthma or cystic fibrosis,  
20 bone disease, such as osteoporosis, joint disease, liver disease, disease of the skin, such as psoriasis or eczema, ophthalmic disease, otolaryngeal disease, other neurological disease such as Turret syndrome, schizophrenia, depression, autism, or stoke, or metabolic disease such as a glycogen storage disease or diabetes. It is to be understood that any disease whereby expression of a particular protein, provision of a therapeutic protein, provision of a drug, inhibition of expression of a  
25 particular protein, etc., which can be accomplished via the use of the delivery systems of this invention and according to the methods of this invention, is to be considered as part of this invention.

In one embodiment, the delivery systems of this invention and methods of this invention treat a condition of the skin, nails, scalp, hands, feet, or combination thereof in the subject. In another  
30 embodiment, the delivery systems of this invention and methods of this invention treat infection, inflammation, eczema, dermatitis, keratosis, urticaria, allergy, acne, folliculitis, furuncles, psoriasis, rosacea, pityriasis, cancer, precancerous lesions, muscular pain, arthritis, heart disease, osteoporosis, osteopetrosis, burns, non-healing wounds, scars, skin ulcers, hyperhydrosis, ichthyosis, lupus of the skin, sun damage, vitiligo, or a combination thereof, in the subject. In another embodiment, the

delivery systems of this invention and methods of this invention treat incorporate an analgesic, an anesthetic, an antioxidant, a growth factor, a hormone, an extracellular matrix component, or a combination thereof, and enable their retention within, and/or permeation of the skin.

5 In another embodiment, the delivery systems of this invention and methods of this invention provide for good skin care, or in another embodiment, prevent or reduce signs of aging. In one embodiment, the delivery systems of this invention and methods of this invention provide for skin retention of a sun screen, or in another embodiment, retention of a moisturizing agent. In another embodiment, the delivery systems of this invention and methods of this invention provide for skin exfoliation, or in  
10 another embodiment, diminishing signs of skin stretching. In another embodiment, the delivery systems of this invention and methods of this invention provide for skin retention of radical scavengers, retinoids, moisturizing agents, vitamins, amino acids, lipids, proteins, enzymes, skin extra cellular matrix components (e.g.; collagen, elastin), or a combination thereof.

15 In another embodiment, the delivery systems of this invention may comprise nanoparticles which may be conjugated to an agent of interest, with the nanoparticles being dispersed within the microemulsions of this invention, or as part of the vehicles of this invention, for use in delivery to the skin. Such nanoparticles will readily penetrate the skin, and may be retained within specific layers of the skin, as, for example, demonstrated herein in Example 7.

20 In one embodiment, this superparamagnetic nanoparticles have a core comprised of an iron ore that is strongly attracted by a magnet. In one embodiment, the nanoparticles comprise magnetite, which in one embodiment refers to a molecule with a general formula of  $\text{Fe}_3\text{O}_4$ , which in another embodiment, possesses a  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  ratio of about 1:1.5 to about 1:2.5, or in another embodiment,  
25 about 1:2. In another embodiment, the superparamagnetic nanoparticles may comprise chemical equivalents of magnetite, such as, for example, and in one embodiment,  $(\text{Fe},\text{M})\text{OFe}_2\text{O}_3$  where M may be, in one embodiment, Zn, Co, Ni, Mn, Cr, Au, or Ag.

In one embodiment, the nanoparticles for use in the systems and according to the methods of this  
30 invention may range in size from 1-100 nm.

Polymers may be conjugated to the nanoparticles, by an array of means, well known by those skilled in the art. The compound of interest, in turn, may be conjugated to the polymer, conjugated to the

nanoparticle, or, as a function of the physico-chemical properties of the compound, it may be directly affixed to the nanoparticle.

In one embodiment, the choice of polymer utilized may be a function of the particles employed. In one embodiment, the polymer may comprise polyacrylic acid, polystyrene sulfonic acid, polyvinyl sulfonic acid, polyethylene oxide, polypropylene oxide, polyvinyl alcohol, photopolymerizable macromers, biodegradable polymers such as but not limited to PLGA, or a combination thereof. In another embodiment, the polymer comprises a surfactant, a polyethylene glycol, a lignosulfonate, a polyacrylamide or a biopolymer. In another embodiment, the biopolymer may comprise polypeptides, cellulose and its derivatives such as hydroxyethyl cellulose and carboxymethyl cellulose, alginate, chitosan, lipid, dextran, starch, gellan gum or other polysaccharides, or a combination thereof.

It is to be understood that the delivery systems of this invention, may comprise any single or combinations of the embodiments listed herein, and comprise what is to be considered part of this invention.

In another embodiment, the delivery systems of this invention and methods of this invention provide a means of treating hair loss, or in another embodiment, diminishing cellulite, or in another embodiment, diminishing signs of aging.

It is to be understood that numerous compounds are known in the art for the treatment or, amelioration of symptoms of, or reduction of pathology of the conditions, diseases and disorders listed hereinabove, and the physico-chemical properties of many of these compounds are well known, such that it will be apparent to one skilled in the art as to how to make use of such compounds in the delivery systems of this invention and formulate them for use according to the methods of this invention, and as such, represent embodiments of this invention.

In other embodiments, the delivery systems of this invention may comprise other components, which provide desired properties to the skin, or increase shelf-life of the product, or a combination thereof. In one embodiment, such components may include, *inter-alia*, preservatives, such as, for example, sodium bisulfite, sodium thiosulfate, ascorbate, benzalkonium chloride, chlorobutanol, thimerosal, phenylmercuric borate, parabens, benzylalcohol and phenylethanol. These agents may be present in amounts of from 0.001 to 5% by weight.

In one embodiment, such components may include, *inter-alia*, buffering agents, such as, for example, alkali or alkali earth carbonates, phosphates, bicarbonates, citrates, borates, acetates, succinates and the like, such as sodium phosphate, citrate, borate, acetate, bicarbonate and carbonate.

5 These agents may be present in amounts sufficient to maintain a pH of the system of between 4 to 8. The buffering agent therefore may be much as 5% by weight of the total composition.

In one embodiment, the delivery systems of this invention may be supplied in a patch form, as will be appreciated by one skilled in the art. In one embodiment, the saturating agent is first applied in  
10 one patch, or in another embodiment, topically, and in another embodiment, the vehicle containing the compound of interest is applied topically, or in another embodiment, at the site, within another patch, or in another embodiment, applied to the initial patch. In one embodiment, a patch may be prepared as described, in US Patent No. 5,503,843. In one embodiment, the patch may be a liquid reservoir patch, such as that disclosed in U.S. Patent 4,829,224 and U.S. Patent 4,983,395, the  
15 disclosures of which are hereby incorporated by reference. Such patches may be constructed to comprise 2 chambers, one in which the saturating agent is first delivered to a skin surface, and at a specified time, the compound of interest is delivered in its appropriate vehicle from a second chamber, at a desired time, thus a singular patch configuration may accommodate delivery of the two components separately. In one embodiment, such a configuration may comprise two chambers, for  
20 delivery of liquid components from the patch, as will be understood by one skilled in the art.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting.

## EXAMPLES

### **Materials and Experimental Methods**

#### ***Reagents***

30 Span 80 (Sp) and Tween 80 (Tw) were obtained from Aldrich (St. Louis, MO, USA); isopropyl myristate (IPM) and phosphate-buffered saline tablets (PBS-T; 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl) from Sigma (St. Louis, MO, USA); 1,2-Octanediol (OCT, 97%) from Lancaster Synthesis (Pelham, NH, USA). [11, 12-<sup>3</sup>H(N)] Retinoic acid (all trans, <sup>3</sup>H-RA, 1 mCi/mL) and D-[2-<sup>3</sup>H] mannitol, (<sup>3</sup>H-MN, 10 mCi/mL) by American Radiolabeled Chemicals, Inc.

(St. Louis, MO, USA) were supplied by NEN<sup>TM</sup> Life Science Products, Inc. (Boston, MA, USA). Scintisafe Plus 50% (Advanced Safety LSC-Cocktail) was purchased from Fisher Chemical (Fair Lawn, NJ, USA). Fresh filtered Milli-Q water (W) was used for any aqueous solution. A phosphate-buffered saline (PBS, pH 7.4) solution was prepared by dissolving 1 PBS-T in 200 mL of Milli-Q water. All chemicals were used as received. Radiolabeled drugs were stored at -20°C. All chemicals and their solutions were always protected from light.

### Vehicle Preparation

Vehicles used in study are as described in Table I.

Table 1: Composition of the microemulsions used

| Composition % (w/w) |    |    |       |      |      | Content (rate %) |                     |
|---------------------|----|----|-------|------|------|------------------|---------------------|
| Emulsion            | W  | I  | T     | S    | O    | Hydrophobic      | Hydrophillic        |
| <b>A</b>            | 1  | 84 | 8.65  | 2.88 | 3.46 | 86.88            | 13.12               |
| <b>B</b>            | 6  | 73 | 12.12 | 4.04 | 4.85 | 77.04            | 22.96 <sub>15</sub> |
| <b>C</b>            | 10 | 60 | 17.31 | 5.77 | 6.92 | 65.77            | 34.23               |
| <b>D</b>            | 10 | 50 | 23.08 | 7.69 | 9.23 | 57.69            | 43.21               |
| <b>E</b>            | 40 | 30 | 17.31 | 5.77 | 6.92 | 35.77            | 64.23               |
| <b>F</b>            | 55 | 20 | 14.42 | 4.81 | 5.77 | 24.81            | 75.19               |

Microemulsions (Me) were prepared as described [B. Baroli, M.A. Lopez-Quintela, , M.B. Delgado-Charro A.M. Fadda J. Blanco-Mendez, Journal of Controlled Release, 69 (2000) 209-218]. Four water-in-oil (w/o) microemulsions (Me A, Me B, Me C, and Me D) were prepared, and two oil-in-water (o/w) microemulsions (Me E and Me F) were formulated. The indicated quantities of water (W), isopropyl myristate (IPM), surfactants [Tween 80 (T) and Span 80 (S)], and the co-surfactant 1,2-octanediol (O) were weighed into glass vials, at amounts which are reflected in the vehicle component ratios listed in Table I. Resulting mixtures were vortexed for 5 minutes. Microemulsions were then left overnight at 60°C under constant stirring, then brought to room temperature in 4-5 hours.

<sup>3</sup>H-mannitol (<sup>3</sup>H-MN) supplemented vehicles were obtained by pouring established vehicle aliquots into drug-containing vials. Mixtures were then stirred for 12 hours at room temperature. <sup>3</sup>H-retinoic acid (RA) supplied in an ethanolic solution (ethanol:water = 9:1), was

allowed to dry overnight in scintillation vials, at which point the vehicle was poured into the drug-containing vial, then stirred for 12 hours at room temperature.

### ***Skin Preparation***

5 Full-thickness excised human cadaver abdominal skin was obtained within 10-20 hours post-mortem (National Disease Research Interchange, Philadelphia, PA, USA). After surgical removal, the skin was cut into 10 x 10 cm<sup>2</sup> pieces, introduced into a plastic bag, and then into a heat-sealed aluminum bag. Samples were immediately frozen at -80 °C, and maintained at this temperature until use. Skin samples were defrosted overnight at 4 °C prior to conducting the diffusion cell experiments. Excess  
10 fat was carefully removed from thawed skin using surgical scissors. Skin was cut into 2 cm<sup>2</sup> pieces, and samples were randomized such that adjacent 2-cm<sup>2</sup> pieces not used. Triplicates were prepared for each sample set. Skin samples were utilized within three months following excision, and within two days following defrosting. Skin samples were heterogeneous with regards to donor sex, age, and race.

15

### ***Permeation Studies and Skin Conductivity Measurements***

Permeation studies were performed at room temperature, in triplicate (n=3). Human cadaver skin samples were clamped in side-by-side diffusion cells (diffusional area: 0.636 cm<sup>2</sup>; volume: 2.4 mL; PermeGear, Riegelsville, PA). Experiments were carried out in static conditions, and with or without  
20 pretreatment of the skin. Skin pretreatment comprised saturating SC with water (W), isopropyl myristate (IPM), surfactants [Tween 80 (T) and Span 80 (S)], and the co-surfactant 1,2-octanediol (O) for 12 hours. In all other cases, skin was hydrated with PBS for one hour before starting the experiment. Vehicles used to saturate or hydrate the skin did not contain the drugs. After the pretreatment or the hydrating phase, vehicles in contact with the skin were carefully removed. The  
25 diffusion cell donor compartments were cleaned, dried with an adsorbing cloth, and subsequently refilled with 2 mL of drug-loaded delivering vehicle (time 0). The diffusion cell receiver compartments remained filled with PBS. Vehicles used for permeation studies on pretreated skin samples are indicated with the following wording: "X>Z", where "X" represents the skin-saturating vehicle, and "Z" the drug delivering vehicle. In contrast, vehicles that were used to deliver <sup>3</sup>H-RA  
30 after the hydrating phase are reported with their own abbreviation (e.g., IPM).

To ensure skin barrier integrity and to quantify its modification upon contact with the pretreatment or donor solutions, skin electrical properties were measured at time -12 h, at time 0 h and after the final sampling (time 18 h) of the diffusion experiments, according to the following protocol. At each data

point, donor compartments were completely sampled, extensively washed with PBS, and then filled with fresh PBS. The current passing through the skin was finally recorded at 10 Hz and 1 kHz with an AC voltage of 100 mV using Ag-AgCl electrodes (E242, In Vivo Metric, Healdsburg, CA, USA), which were connected to an AC signal generator (HP 33120A, 15 MHz, Function Arbitrary Waveform Generator, Hewlett Packard, Palo Alto, CA, USA) in series with a digital meter (Radio Shack, Forth Worth, TX, USA). Receiving compartments were subjected to the washing and refilling procedure only at time 18h.

The skin barrier was considered intact if its measured resistance provided values between 12-120 kΩcm<sup>2</sup>, DC (ref: Inada H, Ghanem A.H., and Huguchi W.I. *Pharm. Res.* **11**, 687-697 (1994).)

Ohm first law describes the relationship between the voltage (Volt; V) and intensity of a current (Ampere, A) flowing in a particular electric circuit, as follows :

$$R = \frac{V}{I}$$

where R represent the “resistance” of the particular circuit.

Resistance is expressed in Ohms (Ω). In terms of specific resistance of a material, (ρ), Ohm second law indicates:

$$R = \rho \frac{l}{s}$$

where *l* is the length and *s* is the area of the resistant material in the circuit. The above equations may be simplified as:

$$\rho = \frac{V}{I} \frac{s}{l}$$

Specific resistance (ρ) is typically expressed as Ω\*m. In this case, the material providing resistance to the flux of ions (which generates the current) is the skin, where *l* is its thickness and *s* its area. Thus skin specific resistance (ρ) is expressed in Ω\*cm<sup>2</sup>.

An AC circuit is characterized by three quantities: V and I that oscillate sinusoidally at a frequency (*f*) equal to 1/T (Hz), where T represents the period of time (in seconds) needed to complete an entire oscillation. Such a circuit generates a resistance that is the resultant of an ohmic, an inductive, and a capacitive contribution, called impedance (Z).

Z can be mathematically expressed by the following equation:

$$Z = R + i\omega L - \frac{i}{\omega C}$$

5

where it is evident that impedance (Z) is influenced by the frequency of oscillation (being  $f = \omega/2\pi$ ) in its inductive ( $i\omega L$ ) and capacitive ( $i/\omega C$ ) components.

Previous reports indicated that the specific impedance of an excised human skin, with intact skin  
 10 barrier properties ranges between  $10 \text{ k}\Omega\cdot\text{cm}^2$  and  $50 \text{ k}\Omega\cdot\text{cm}^2$  (K. Konturi, et al. *Pharm Res* 10(3): 381-385, 1993), with impedance calculated via Ohm's second law, assuming the frequency of oscillation is low, due to the minimal contribution of the inductive and capacitive contributions.

Under these conditions specific impedance and specific resistance are synonymous, and a  
 15 resistance of  $10 \text{ k}\Omega\cdot\text{cm}^2$ , at AC 100 mV and 10 Hz, results in a maximum current of:

$$\rho = \frac{V}{I} \frac{s}{l} \Rightarrow I = \frac{V}{\rho} s = \frac{100 \cdot 10^{-3} \text{ V}}{10 \cdot 10^3 \Omega \text{ cm}^2} 0.636 \text{ cm}^2 = 6.36 \mu\text{A}$$

where V and I are at the maximum value of their oscillating phases. Using the same equation, a  
 current of  $1.27 \mu\text{A}$  should be expected for skin resistance of  $50 \text{ k}\Omega\cdot\text{cm}^2$

20

Therefore the skin barrier was considered intact if its initial current readings fluctuated between  $1.27$   
 and  $6 \mu\text{A}$  at 10 Hz. Nevertheless, current flow through the skin was always examined at 10 Hz and 1  
 kHz frequency. It has been reported that skin impedance decreases by increasing the frequency of  
 the AC, and that a decreased skin impedance reflects a major flux of ionic species across the skin  
 25 (higher conductivity) either by pore formation or uniform lipid fluidization. Consequently, at 1 kHz,  
 it was possible to monitor modifications of the skin barrier integrity, resulting from exposure to the  
 vehicles described above, which were not possible to be observed at 10 Hz.

During the diffusion experiments, receiver compartments were completely sampled at specific time-  
 30 points, and then replenished with fresh PBS. Sampled solutions were placed into scintillation vials  
 (VWR Scientific Products, West Chester, DA) and analyzed for drug content, as described below.  
 For discussion purposes, transport data are sometimes reported as ratio of the total amount of drug  
 retained in the skin versus the total amount of drug permeated through the skin (also indicated as R/P



ratio). Therefore, vehicles that promoted drug retention will show a R/P ratio expressed by a number bigger than 1, whereas vehicles that hindered retention yielded ratios smaller than 1. Permeability coefficients were calculated at steady state according to the following equation:

$$P = \frac{dQ}{dt} \frac{1}{CdA} \quad (6)$$

- 5 where  $\frac{dQ}{dt}$  represents drug flux at steady state condition (g/h),  $Cd$  is the drug concentration in the delivering vehicle (g/cm<sup>3</sup>), and  $A$  is the diffusion area (cm<sup>2</sup>).

Permeability coefficients are reported in Table III and IV, for formulations comprising retinoic acid and mannitol, respectively, and are expressed in cm/h.

10

Table III: Permeability coefficients for the conditions tested with retinoic acid (<sup>3</sup>H-RA)

|             | X                 | SD                |               | X                 | SD                 |               | X                 | SD                |
|-------------|-------------------|-------------------|---------------|-------------------|--------------------|---------------|-------------------|-------------------|
| <b>W</b>    | $7.5 \times 10^4$ | $1.7 \times 10^4$ | <b>W&gt;W</b> | $2.8 \times 10^3$ | $2.7 \times 10^4$  |               |                   |                   |
| <b>O</b>    | $2.6 \times 10^4$ | $6.8 \times 10^4$ | <b>O&gt;W</b> | $8.5 \times 10^5$ | $1.81 \times 10^6$ | <b>W&gt;O</b> | $2.1 \times 10^4$ | $6.0 \times 10^5$ |
| <b>T</b>    | $3.8 \times 10^4$ | $7.8 \times 10^5$ | <b>T&gt;W</b> | $4.4 \times 10^5$ | $1.3 \times 10^6$  | <b>W&gt;T</b> | $6.6 \times 10^4$ | $3.5 \times 10^5$ |
| <b>S</b>    | $2.2 \times 10^4$ | $2.6 \times 10^7$ | <b>S&gt;W</b> | $2.7 \times 10^5$ | $4.4 \times 10^6$  | <b>W&gt;S</b> | $4.0 \times 10^4$ | $5.7 \times 10^5$ |
| <b>I</b>    | $4.9 \times 10^4$ | $3.6 \times 10^5$ | <b>I&gt;W</b> | $4.3 \times 10^5$ | $2.7 \times 10^7$  | <b>W&gt;I</b> | $2.8 \times 10^3$ | $6.3 \times 10^4$ |
| <b>Me.A</b> | $4.5 \times 10^4$ | $4.2 \times 10^5$ | <b>I&gt;O</b> | $1.3 \times 10^4$ | $1.2 \times 10^5$  | <b>O&gt;I</b> | $3.9 \times 10^4$ | $6.2 \times 10^5$ |
| <b>Me.B</b> | $3.7 \times 10^4$ | $1.8 \times 10^5$ | <b>I&gt;T</b> | $2.7 \times 10^4$ | $1.2 \times 10^5$  | <b>T&gt;I</b> | $2.3 \times 10^4$ | $5.0 \times 10^5$ |
| <b>Me.C</b> | $4.0 \times 10^4$ | $1.5 \times 10^5$ | <b>I&gt;S</b> | $4.4 \times 10^5$ | $3.4 \times 10^6$  | <b>S&gt;I</b> | $1.1 \times 10^4$ | $1.1 \times 10^5$ |
| <b>Me.D</b> | $2.4 \times 10^4$ | $2.0 \times 10^5$ | <b>O&gt;T</b> | $3.1 \times 10^4$ | $5.5 \times 10^6$  | <b>T&gt;O</b> | $1.3 \times 10^4$ | $6.6 \times 10^6$ |
| <b>Me.E</b> | $2.7 \times 10^4$ | $1.0 \times 10^4$ | <b>O&gt;S</b> | $1.2 \times 10^4$ | $2.7 \times 10^6$  | <b>S&gt;O</b> | $9.8 \times 10^5$ | $9.7 \times 10^6$ |
| <b>Me.F</b> | $2.2 \times 10^4$ | $5.5 \times 10^6$ | <b>S&gt;T</b> | $1.4 \times 10^4$ | $2.4 \times 10^5$  | <b>T&gt;S</b> | $8.2 \times 10^5$ | $8.9 \times 10^6$ |

Table III: Permeability coefficients for the conditions tested with mannitol (<sup>3</sup>H-MN)

|          | X                 | SD                |               | X                 | SD                |               | X                 | SD                |
|----------|-------------------|-------------------|---------------|-------------------|-------------------|---------------|-------------------|-------------------|
| <b>W</b> | $2.2 \times 10^4$ | $3.4 \times 10^5$ | <b>W&gt;W</b> | $5.7 \times 10^4$ | $3.5 \times 10^4$ |               |                   |                   |
| <b>O</b> | $1.3 \times 10^4$ | $8.8 \times 10^5$ | <b>O&gt;W</b> | $2.2 \times 10^4$ | $1.1 \times 10^5$ | <b>W&gt;O</b> | $1.4 \times 10^4$ | $3.1 \times 10^4$ |
| <b>T</b> | $7.3 \times 10^5$ | $6.3 \times 10^7$ | <b>T&gt;W</b> | $3.4 \times 10^4$ | $4.0 \times 10^5$ | <b>W&gt;T</b> | $5.6 \times 10^4$ | $1.4 \times 10^4$ |

|             |                   |                   |               |                   |                   |               |                   |                   |
|-------------|-------------------|-------------------|---------------|-------------------|-------------------|---------------|-------------------|-------------------|
| <b>S</b>    | $7.6 \times 10^4$ | $4.8 \times 10^4$ | <b>S&gt;W</b> | $1.0 \times 10^4$ | $5.0 \times 10^6$ | <b>W&gt;S</b> | $4.1 \times 10^4$ | $1.7 \times 10^5$ |
| <b>I</b>    | $9.2 \times 10^2$ | $1.3 \times 10^2$ | <b>I&gt;W</b> | $2.8 \times 10^4$ | $6.0 \times 10^5$ | <b>W&gt;I</b> | $1.5 \times 10^2$ | $3.0 \times 10^3$ |
| <b>Me.A</b> | $2.1 \times 10^4$ | $5.3 \times 10^5$ | <b>I&gt;O</b> | $3.9 \times 10^4$ | $1.0 \times 10^5$ | <b>O&gt;I</b> | $1.3 \times 10^2$ | $8.0 \times 10^4$ |
| <b>Me.B</b> | $5.5 \times 10^4$ | $4.2 \times 10^4$ | <b>I&gt;T</b> | $7.4 \times 10^5$ | $2.6 \times 10^5$ | <b>T&gt;I</b> | $8.6 \times 10^4$ | $9.8 \times 10^5$ |
| <b>Me.C</b> | $5.2 \times 10^4$ | $5.6 \times 10^4$ | <b>I&gt;S</b> | $9.2 \times 10^6$ | $4.5 \times 10^7$ | <b>S&gt;I</b> | $1.1 \times 10^4$ | $2.3 \times 10^5$ |
| <b>Me.D</b> | $1.1 \times 10^4$ | $1.1 \times 10^5$ | <b>O&gt;T</b> | $7.1 \times 10^4$ | $2.8 \times 10^5$ | <b>T&gt;O</b> | $3.6 \times 10^4$ | $2.0 \times 10^5$ |
| <b>Me.E</b> | $1.3 \times 10^4$ | $1.7 \times 10^5$ | <b>O&gt;S</b> | $3.7 \times 10^4$ | $5.4 \times 10^5$ | <b>S&gt;O</b> | $3.7 \times 10^4$ | $1.1 \times 10^4$ |
| <b>Me.F</b> | $8.8 \times 10^5$ | $2.1 \times 10^5$ | <b>S&gt;T</b> | $2.7 \times 10^4$ | $3.6 \times 10^5$ | <b>T&gt;S</b> | $3.9 \times 10^5$ | $2.3 \times 10^5$ |

These values reflect the ease with which the model drugs diffuse across the skin.

### ***Drug Quantification***

- 5 Drug concentrations were measured using a liquid scintillation analyzer, (Packard Instrument Company, Inc. Downers Grove, IL, USA; Model: A2020) using 10 mL of Scintisafe.

### ***Statistics***

- Data were analyzed by the two-population Student's *t*-Test (value of significance  $p < 0.05$ ). Unless  
10 clearly stated, data are always reported as mean  $\pm$  SD.

## **EXAMPLE 1**

### **Microemulsion Drug Delivery Systems Promote Drug Retention In and/or Permeation Through Skin as a Function of their Physico-Chemical Properties**

15

Oil in water and water in oil microemulsions comprising a drug were tested for their relative effects on drug retention within and permeation through skin. Retinoic acid was used as a model lipophilic drug to explore these effects, as described hereinbelow.

- 20 A ternary phase diagram of the microemulsion system and phase characterization is shown in Figure 1, and the physico-chemical properties of microemulsion components are displayed in Table 3. Retinoic acid was utilized as a model lipophilic molecule, for the drug delivery studies.

Table 3:

|  | <b>MW</b> | <b>HLB</b> | <b>Log KO/W</b> | <b>PB</b> | <b>Visc.</b> |
|--|-----------|------------|-----------------|-----------|--------------|
|--|-----------|------------|-----------------|-----------|--------------|

|           |               |     |                 |                   |            |
|-----------|---------------|-----|-----------------|-------------------|------------|
| <b>W</b>  | 18.02         |     | n.a.            | n.a.              |            |
| <b>I</b>  | 270.46        |     | $6.40 \pm 0.31$ | $98.07 \pm 6.02$  | $< 0.0034$ |
| <b>T</b>  | $\sim 1310^*$ | 15  | $1.69 \pm 2.41$ | $39.39 \pm 10.99$ | 0.29-0.46  |
| <b>S</b>  | 428.62        | 4.3 | $3.73 \pm 0.88$ | $88.49 \pm 4.41$  | $\sim 1$   |
| <b>O</b>  | 146.23        | 6-9 | $1.78 \pm 0.31$ | $46.70 \pm 4.65$  | $< 0.043$  |
| <b>RA</b> | 300.44        |     | $4.89 \pm 0.94$ | $98.99 \pm 1.33$  |            |

Molecular weights (MW) of water (W), isopropyl myristate (I), Tween 80 (T), Span 80 (S), 1,2-octanediol (O), trans retinoic acid ( $^3\text{H}$ -RA) are given in Da. Mn is the average MW. HLB is the hydrophilic-lipophilic balance value. Log Ko/w (octanol/water partition coefficient) and PB (protein binding prediction) were calculated using *CSLogP<sup>TM</sup>* and *CSLogP<sup>TM</sup>* available, as a limited free trial, at <http://www.chemsilico.com>. Viscosity values are given in Pa\*s. "O" was considered a hydrophilic molecule due to its Log Ko/w and PB values

In order to determine drug retention versus permeation in skin, the skin barrier integrity must be evaluated. Skin sample integrity is assessed, as depicted in Figure 2. Skin permeability coefficients for retinoic acid were determined using this experimental setup, according to the equation as described hereinabove, in the methods. The nomenclature to establish hydration with PBS, followed by drug treatment in vehicle Z is denoted by "Z", whereas skin pre-saturated with vehicle "X", then 12 hours later treated with a drug in vehicle Z is denoted by "X>Z".

The experimental setup enabled measurement of trans retinoic acid penetration through and retention within the skin using microemulsions. Figure 3A demonstrates skin resistivity determinations, wherein most microemulsion vehicle pretreatment did not appreciably alter skin resistivity, regardless of whether retinoic acid was present in the formulation.

Measurement of retinoic acid penetration within skin showed considerable variability, as a function of which microemulsion was utilized, with greater skin permeation for water-in-oil emulsions, when in a more lipophilic formulation. RA retention within the skin, similarly was significantly greater in water-in-oil emulsions, when in a more lipophilic formulation, however, oil-in-water microemulsions showed greater retention, when  $^3\text{H}$ -RA was in a more hydrophilic formulation (Figure 3B, D, E & F).

While the A, B and C microemulsions delivered a roughly 30 % greater amount of  $^3\text{H}$ -RA in comparison with other microemulsions, the delivery did not correlate with microemulsion physico-chemical properties or skin resistivity, with retention versus permeation seemingly increased once a particular hydrophilic content threshold of the microemulsion is achieved.

5

In order to determine whether the above phenomenon reflected an influence of the drug composition, a hydrophilic drug, radiolabeled ( $^3\text{H}$ ) mannitol ( $^3\text{H}$ -MN) was delivered in the microemulsions used for  $^3\text{H}$ -RA delivery.

10 Some physico-chemical characteristics of mannitol are displayed in Table 4:

|    | MW     | Log KO/W | PB   |
|----|--------|----------|------|
| MN | 182.17 | -2.24    | 4.79 |

Figure 4 demonstrates skin resistivity determinations, wherein most microemulsion vehicle pretreatment did not appreciably alter skin resistivity, regardless of whether mannitol was present in the formulation. Mannitol was retained in skin at higher levels than retinoic acid, in all microemulsion formulations tested, though the retention versus permeation trends were consistent with that obtained for  $^3\text{H}$ -RA. Permeation profiles for mannitol however, indicate an enhancer effect for mannitol.

20

## **EXAMPLE 2**

### **Drug Retention In and/or Permeation Through Skin as a Function of the Physico-Chemical Properties of Particular Components of the Microemulsions**

25 Specific components of the microemulsions differentially affect trans retinoic acid penetration through the skin. Vehicles used to deliver trans retinoic acid through the skin were water (W), 1,2-octanediol (O), Tween 80 (T), Span 80 (S), and isopropyl myristate (I). While the microemulsion components were found to alter ion transport through the skin, resistivity was only found to be affected by the use of Span (Figure 5). Skin retention of  $^3\text{H}$ -RA was evident when delivered in  
30 water, 1,2-octanediol or Span 80, while its delivery in Tween 80 or isopropyl myristate resulted in

greater permeation. The high retention of  $^3\text{H}$ -RA when delivered in water may in fact be due to a partition event.

An inverse relationship was found between the vehicle molecular weight and the total amount of  $^3\text{H}$ -RA that entered and was retained in the skin (Figure 6), with a linear relationship between vehicle molecular weight and the total amount of  $^3\text{H}$ -RA that permeated through the skin, though water deviated significantly from this, possibly as a reflection of the "weight" of an RA-W partition event.

Similarly, components of the microemulsions differentially affected retention versus permeation of mannitol in skin, with water, 1,2-octanediol and Span 80 favoring retention, while isopropyl myristate and Tween 80 favoring permeation (Figure 7). Great variability in the total cumulative amounts (R, P, R+P) made any correlation between the vehicle's molecular weights, Log Ko/w, PB values, and  $^3\text{H}$ -MN permeability coefficients uninterpretable (Figure 8).

Comparing the data obtained for retinoic acid and mannitol, the following tentative conclusions regarding drug transport may be drawn:

When water is the vehicle for the drug, aqueous pores created enable transport of both  $^3\text{H}$ -MN and RA, with the latter potentially having a strong partitioning effect in the stratum corneum lipids. When Octanediol is the vehicle, micelle formation may occur, with the drug potentially located in the micelle core. When Span 80 is the vehicle, micelle formation may similarly occur, with the drug potentially located in the micelle core. When isopropyl myristate is the vehicle, a partitioning effect in the stratum corneum may occur with mannitol, and matrix disorganization, stratum corneum fluidization may occur for retinoic acid transport.

### **EXAMPLE 3**

#### **Drug Retention In and/or Permeation Through Skin as a Function of the Particular Components of the Microemulsions, When Skin is Previously Hydrated with Water**

Figures 9 and 10 demonstrate that some of the profiles obtained in Example 2 change, in regard to  $^3\text{H}$ -RA retention when delivered in vehicles consisting of specific components of the microemulsions, when the skin is previously hydrated with water.

While saturation with water did not alter the retention versus permeation profile, the ratio was enhanced in samples hydrated previously, as compared to not, when  $^3\text{H}$ -RA was delivered in water. Moreover, hydration lowered the ratio in  $^3\text{H}$ -RA supplied in the Tween 80 vehicle, as compared to  
5 non-hydrated samples. Similarly, permeation as a whole was increased in  $^3\text{H}$ -RA supplied in both water and isopropyl myristate vehicles.

The effects exerted by prior skin hydration may be a reflectance of the associated substratum corneum (SC) swelling upon contact with water. Such contact may result in increased size of  
10 corneocytes, larger distance between corneocytes, easier drug partitioning in the intercellular space, easier drug permeation through the polar route, or a combination thereof. Hydration followed by water vehicle delivery of  $^3\text{H}$ -RA enables ready partitioning, by both polar and apolar routes, whereas isopropyl myristate vehicle delivery enables partitioning of the drug via the apolar route, since both drug and vehicle are hydrophobic.

15 In order to determine whether similar effects of hydration occur when a hydrophilic drug is used, mannitol delivery under similar conditions was tested (Figures 11 and 12). Skin saturation with water changed the mannitol retention versus permeation profiles. Hydration followed by delivery in water increased this ratio, when compared to water delivery alone, and similarly hydration followed  
20 by isopropyl myristate delivery, as opposed to delivery in the latter alone. Hydration followed by delivery in 1, 2, octanediol or Span resulted in a diminished ratio, in comparison to delivery in the vehicles alone, however.

Hydration followed by Tween or Span vehicle delivery of mannitol resulted in enhanced skin  
25 resistivity, while when 1,2 octanediol is the delivery vehicle, skin resistivity decreased with time.

When comparing mannitol versus retinoic acid delivery in hydrated skin samples, water as a delivery vehicle resulted in greater entrance of hydrophilic drugs, which may enter through the swelling of the stratum corneum, formation of aqueous pores in skin. Lipophilic drugs may enter via skin  
30 partitioning effect.

When 1, 2-octanediol is the delivery vehicle, both hydrophilic and lipophilic drugs may enter as micelles, the former via partitioning in aqueous pores in the skin, the latter via partitioning in the

stratum corneum lipids. Both Tween and Span as delivery vehicles provide less retention of the drugs within skin, regardless of the nature of the drug, when skin is first hydrated.

#### **EXAMPLE 4**

##### **Drug Retention In and/or Permeation Through Skin as a Function of Skin Saturation with Particular Components of the Microemulsions, When the Drug Delivery Vehicle is Water**

Since hydration exerted some effects on the retention versus permeation values obtained for drug delivery, at least in some instances, as a function of the physico-chemical property of the vehicle, the reverse was then pursued. Skin samples were saturated with specific components of the microemulsions, and drugs were delivered in water alone. Figure 13 demonstrates that the retention versus permeation profiles obtained in Example 3 change, in that retention was high in most groups tested, and diminished permeation was seen.

Such reduction in permeation of the drug may reflect solidification/stabilization of the lipophilic lamellae; creation of a different hydrophilic-hydrophobic balance inside the SC; amphiphilic surfactant molecules in the SC lipophilic lamellae favor the partitioning of drug loaded water in the lipophilic lamellae sequestering drug from permeation, or a combination thereof.

Similar experiments were conducted for mannitol, in order to determine whether the retention versus permeation profile altered when water was the delivery vehicle, and the skin was previously saturated with the respective components (Figure 14 and 15).

Skin saturation with Me components favored retention in all cases. However, skin saturation with surfactant resulted in less retention, as compared to that of water saturation. Whereas with retinoic acid, when the saturating vehicle molecular weight increased (O, T and S samples), the drug retention and skin permeation of the compound decreased, retention increased, when mannitol was delivered. It may be that surfactants form micelle-like structures inside stratum corneum lipids, and the retention versus permeation ratio is reflected as a balance between perturbation of the stratum corneum and the lipophilicity – hydrophilicity balance of the micelle like structure.

#### **EXAMPLE 5**

**Drug Retention In and/or Permeation of Skin as a Function of Skin Saturation with Isopropyl Myristate, or its Use as a Delivery Vehicle**

Since saturation of the skin affected the retention versus permeation profile, when the drug was delivered in water, it was of interest to determine whether saturation with a particular component affects the profile, with the delivery vehicle consisting of these components.

Figure 13 indicated that  $^3\text{H}$ -RA retention was quite high in samples, when skin was previously saturated with isopropyl myristate. It was therefore of interest to determine whether saturation with isopropyl myristate and delivery in other microemulsion components provided greater retention (Figures 16 & 17). Toward this end, samples were saturated with I, and  $^3\text{H}$ -RA was delivered in the respective components, with the various parameters evaluated.

Isopropyl myristate (I) saturation tended to decrease skin resistivity, and the permeation profile of I saturation of skin delivered  $^3\text{H}$ -RA in a Tween vehicle differed greatly from Tween alone, water hydration, Tween delivery, and Tween saturation, water delivery (Figure 16). I saturation phase may provide (1) a more lipophilic skin, (2) a larger lipophilic space within the stratum corneum, and hence a diminished space for the polar route, (3) a reorganization of lipids within the SC lamellas, which could contribute to increase tortuosity and disorganization of aqueous pores. Changes in skin resistivity in I saturated samples, with O and T as the delivery vehicle may reflect that O and T extracted the saturating vehicle (I) and/or skin components by means of partitioning; that O and T co-permeated with  $^3\text{H}$ -RA easier following I saturation of the skin, and/or that O permeation might bring a possible solidification/stabilization of the lipophilic matrix, which could justify higher retentions, while T permeation might create alternative routes of permeation. Because a linear relationship was found between increasing molecular weight and permeation values, it seems likely that the surfactants use an apolar route to co-transport  $^3\text{H}$ -RA in skin.

When mannitol delivery was evaluated under these conditions (Figure 18) the retention versus permeation profile increased with I saturation, and permeation profiles as in  $^3\text{H}$ -RA, were enhanced with I saturation, when Tween served as the delivery vehicle. A similar linear relationship between increased vehicular molecular weight, and mannitol values.

$^3\text{H}$ -RA delivery in isopropyl myristate showed some differences, when the skin was previously saturated with the respective microemulsion components. Skin saturation with surfactants favored



retention, this despite the permeation-favoring nature of isopropyl myristate, the delivery vehicle. Increasing vehicular molecular weight resulted in increased permeation, probably reflecting that larger molecules may penetrate the stratum corneum, however  $^3\text{H}$ -RA permeation will result, due to architectural disturbance of the skin layer.

Mannitol delivery in isopropyl myristate was similarly evaluated. As with  $^3\text{H}$ -RA, saturation with surfactants favored retention, despite delivery in I. In both cases,  $^3\text{H}$ -RA and mannitol, saturation with 1, 2-octanediol or Span appears to create micelles and an overall drug retention effect is favored, probably due to partitioning effects within the skin.

#### **EXAMPLE 6**

##### **Drug Retention In and/or Permeation of Skin as a Function of Skin Saturation with Combined Surfactants, or their Use as a Delivery Vehicle**

It was of interest to determine whether saturation with the surfactants affects the retention versus permeation profile for  $^3\text{H}$ -RA delivery. Toward this end, skin was saturated with 1,2-octanediol (O), Tween 80 (T), or Span 80 (S), and  $^3\text{H}$ -RA skin delivery in the other 2 respective surfactants was evaluated (Figure 21). Best results were obtained when O was the delivery vehicle, and Span 80, or Tween 80 were used to saturate the skin, though O saturation of the skin, followed by delivery in Span 80 or Tween 80 provided good retention versus permeation profiles. Only Span/Tween combinations, saturation or delivery, favored permeation.

When mannitol delivery was evaluation, all combinations favored mannitol retention, with the exception of Span/Tween combinations, saturation or delivery, which favored permeation, as was the case for retinoic acid delivery.

While on the whole, retinoic acid was retained better within skin, in comparison to mannitol, specific design of vehicle application significantly alters retention versus permeation profiles. If it is assumed that microemulsions are transported through the skin as a whole, in other words as drops surrounded by surfactant and a layer of external phase, then water-in-oil microemulsions partition through the apolar route, while oil-in-water microemulsions partition through aqueous pores.

Water-in-oil microemulsions will likely have most of the mannitol thus delivered, in a hydrophilic inner-core, while retinoic acid will be in the external phase. This structural distinction may reflect the greater retention of mannitol, as compared to  $^3\text{H}$ -RA.

Oil-in-water microemulsions will result in mannitol location in the external phase, whereas retinoic acid is in the internal lipophilic core. It is possible that the external phase hydrates the skin, expands the polar region and causes aqueous pores to be more accessible (tendency to decrease skin resistivity).

In both  $^3\text{H}$ -RA and mannitol delivery, drug retention in the skin increased as the hydrophilic content increased suggesting that the more expanded the aqueous pores the more drug which may enter the skin, while permeation may be controlled by how intact is the skin barrier. While  $^3\text{H}$ -RA was retained better than mannitol when the skin was saturated with specific microemulsion components, mannitol was retained better than  $^3\text{H}$ -RA, when the microemulsions were considered as a whole.

The combination of lipophilic and hydrophilic molecules, as well as the chemical pretreatment order, play important roles in creating skin conditions, which favor drug retention and/or permeation.

## EXAMPLE 7

### Nanoparticles Penetrate the Skin Barrier

#### Materials and Methods

Magnetite and iron nanoparticles were synthesized using a microemulsion (Me) as described (M.A. López Quintela and J. Rivas, "Chemical reactions in microemulsions: A powerful method to obtain ultrafine particles". J. Colloid Interface Sci. 158, 446-451(1993).

Briefly, maghemite nanoparticles were obtained by quickly mixing two microemulsions composed by cyclohexane/Brij-97/aqueous solution of iron salts (0,5 M in  $\text{Fe}^{3+}$ , 1M in  $\text{Fe}^{2+}$  and 0,1 M in HCl) (Me1) and cyclohexane/Brij-97/deionized water/ cyclohexylamine (Me2), which were then maintained under stirring and at 65 °C for 10 minutes.

Nanoparticle formation was indicated by the appearance of a black color in the reacting mixture, to which acetone was then added to allow nanoparticle precipitation. Subsequently, nanoparticles were

isolated by magnetic separation and then washed twice with acetone and deionized water (pH 7). Finally, to obtain a stable dispersion, particles were washed in nitric acid (1 M) and deionized water (pH 7) and then dispersed in a TMAOH solution (0.01 M to 0.1 M) under vigorous stirring. Maghemite nanoparticles were characterized by X-ray electron diffraction (Philips PW-1710), TEM  
 5 (JEOL JEM-2010 operating at 200-kV) and DLS, by determining their Zeta potential and isoelectric point (Malvern Zetasizer Nano ZS, Malvern Instruments), and by magnetic measurements (DMS 1660 vibrating sample magnetometer, VSM)

Iron nanoparticles were instead prepared by mixing two microemulsions, which contained  
 10 AOT/Heptano/ $\text{FeCl}_2$ /deionized water (Me1) and AOT/Heptano/ $\text{NaBH}_4$ /deionized water (Me2), for 15 min. under  $\text{N}_2$ . Stirring allowed Me's droplet to coalesce and a contemporaneous reduction of  $\text{Fe}^{2+}$  to  $\text{Fe}^0$ . Iron nanoparticles were then centrifuged and washed with acetone and dried in an argon-controlled environment. Iron nanoparticles were characterized by TEM (JEOL JEM-2010 operating at 200-kV), DLS, TGA and magnetic measurements (M.A. López-Quintela, J. Quibén and J. Rivas,  
 15 "Use of microemulsions in the production of nanostructured materials". "Industrial Applications of Microemulsions", edited by C. Solans and H. Kunieda, (Surfactant Science Series, Marcel Dekker, N.Y.) pp.247-265, 1997).

For both nanoparticles, DLS measurements were performed using an ALV SP-86 goniometer, ALV  
 20 5000 Multi-tau correlator and a Coherent DPSS 532 laser operating at a wavelength of 532 nm and a power of 100 mW. The correlation functions were accumulated for 2 minutes and analysed using the ALV-5000 WIN software based on the CONTIN algorithm adapted to the specific correlator noise. The temperature was fixed at 20°C. The logarithmically sampled relaxation time spectra (Amplitude vs.  $\log(\tau)$ ), which were the result of the CONTIN inversion of the normalized correlation functions,  
 25 were transformed using the Stokes-Einstein relation, the electrolyte solvent viscosity  $\eta_0$  and refractive index  $n$  at the actual temperature  $T$  in order to yield the hydrodynamic radius  $R_H = (kTq^2\tau)/6\pi\eta_0$  where  $k$  is the Boltzmann constant and  $q = (4\pi n/\lambda)\sin(\theta/2)$  the scattering vector as function of wavelength in vacuum,  $\lambda$ , and scattering angle  $\theta$ .

30 Skin preparation. Full-thickness human skin was obtained from female donors undergoing surgical reduction of abdomen. Anesthesia was induced by Diprivan and maintained with a mixture of Sevoflurane (sevoflurane) and Remifentanyl. Abdominal area was cleaned with a Betadine-soaked gauze, prior to be removed using a cauterizing knife. Excised skin was immediately brought to the

laboratory (c.a. 20 min.; 10°C), where it was cleaned from fats using surgical scissors. Skin was then stored at -23°C until use. Experiment protocols and skin donation were approved by the ethical committee of Azienda Ospedaliera G. Brotzu (Cagliari, Italy; protocols n. 133 and n. 135 respectively issued on 12. 22. 2003 and on 01. 26. 2004). Nevertheless, patient consensus remained  
5 obligatory for specimen donation.

Diffusion experiment. SPION diffusion experiments (n: 3, 4) were carried out using handmade vertical diffusion cells (Rofarma Italia S.r.l., Gaggiano, MI, IT; d:  $9.05 \pm 0.46$  mm, A:  $0.64 \pm 0.03$  cm<sup>2</sup>), which were thermostated at 37 °C (Grant Instruments Ltd., Cambridgeshire, GB). Skin was  
10 defrosted overnight at 4°C, opportunely cut in circular pieces (d: 2.5 cm), randomized, and clamped between donor and receptor chambers of the diffusion cells. Receptor chambers were filled with 7 mL of a PBS solution (pH 7.4). Donor chambers contained instead 200 µL of SPION dispersion or blank solution. Skin pieces were hydrated with PBS for one hour (started at time -1) before beginning the permeation experiments (time 0). At 3, 6, 12 and 24 h receptor chambers were emptied  
15 and solutions analyzed for SPION content. Donor solutions were removed, and skin was washed twice with PBS before unclamping diffusion cells. Finally, tested skin pieces were processed for microscope visualization.

Skin belonging to 30 and 64 years-old donors was respectively used for TMAOH- and AOT-SPION  
20 diffusion experiments. Therefore, skin barrier integrity was assessed by calculating its specific resistivity using Ohm's law before (time -1 and 0) and after (time 3, 6, 12 and 24) each diffusion experiment. Hence, the amount of current crossing the skin was recorded at 10 Hz and 1 kHz with an AC voltage of 100 mV using Ag-AgCl electrodes (E242, In Vivo Metric, Healdsburg, CA, USA), which were connected to an AC signal generator (HP 33120A, 15 MHz, Function Arbitrary  
25 Waveform Generator, Hewlett Packard, Palo Alto, CA, USA) in series with a digital meter (HT58 multi-display multimeter, HT Italia S.r.l., Faenza, RA, IT). SPION concentration in both donor and receptor suspensions were quantified using a CCD simultaneous ICP-OES (VISTA-MPX, Varian Inc., Palo Alto, CA, USA). Prior to be analyzed, samples were diluted with a 1% HNO<sub>3</sub> (Fluka; cat n. 84385) solution. Data were statistically analyzed by unpaired two-population Student t-Test, where a  
30  $p \leq 0.05$  was considered significative.

Skin fixation protocols for microscope observation. Tested skin was processed differently depending on the type of microscope to be used. At the end of the diffusion experiments, each circular specimen

was cut along its diameter in two halves. One half was frozen at  $-23^{\circ}\text{C}$ , whereas the other half was cut in 1-2 mm<sup>3</sup> pieces and fixed for TEM observation (see below).

Frozen skin samples were cut, perpendicularly to the SC surface, in 10 and 16  $\mu\text{m}$  sections using a cryostat (Microm HM 560, Bio-Optica, Milano, IT). Thinner sections were fixed with 4% paraformaldehyde phosphate-buffered solution (Immunofix; Bio-Optica, Milano, IT) for 10 min, stained with a diluted (1:1, distilled water) Mayer hematoxylin solution (Emalum Mayer, Bio-Optica, Milano, IT), and mounted in glycerol gel (Sigma). Specimens were observed using a LTM (Axioplan 2 imaging, Carl Zeiss S.p.A., Arese, MI, IT). Thicker sections were instead deposited onto poly-lysine-coated cover glasses, and dried in a desiccator. Specimens were then deposited on aluminum stubs (Hitachi) and successively coated with a 10 nm carbon layer using a carbon vacuum evaporator (Emitech K950, Emitech Ltd., Ashford, England, GB). Finally, samples were observed using a scanning electron microscope (HR-SEM, Hitachi S-4000, Hitachi, Schaumburg, IL, USA) equipped with X-ray microanalyzer (energy dispersion spectrometry, EDS; Noran, model IFPS, Thermo Noran, Middleton, WI, USA) and a data acquisition software (Kevex, Sigma-32). SEM-EDS sample analysis produced images from backscattered (BSE) and secondary (SE) electrons, and X-ray-generated microanalysis spectra.

Unfrozen skin specimens (1-2 mm<sup>3</sup>) were fixed in 1.5 mL TEM buffer (cacodylate buffer 0.15 M, paraformaldehyde 1%, glutaraldehyde 1.25%) for 4 h and then washed in 2 mL cacodylate- sucrose (CS) buffer (cacodylate buffer 0.1M, sucrose 3.5%, pH 7.2) for 2 h. CS buffer was completely changed after 60 min. of incubation. Pieces were then stored in fresh CS buffer at  $4^{\circ}\text{C}$  before continuing sample preparation protocol. Samples were successively fixed in the minimum amount of osmium tetroxide (2%, water) for 1 h at  $4^{\circ}\text{C}$ , and successively washed in bi-distilled water (3 x 2 min). Samples were then left overnight in an uranyl acetate solution (0.25%, water). The next day, specimens were accurately washed in bi-distilled water (3 x 15 min) prior to start their dehydration, which was accomplished by placing samples in acetone solutions of increasing concentrations (50, 70, 80, 90, 100%) for 15 min. Next, samples were placed in toluene (2 x 30 min), and then in a 1:1 mixture of 'toluene:Epon-resin', where they were left for 24 h, under stirring. Samples were then dried from exciding traces of infiltrating mixture and finally placed inside the embedding-resin-filled molds. Resin was let polymerize at  $60^{\circ}\text{C}$  in an oven for 24h. Embedding resin (a mixture of Epon 44.44 %, dodecenyl succinic anhydride (DDSA) 35.56 %, methyl nadic anhydride (MNA) 17.78 %, benzyl dimethyl amine (BDMA) 2.22 %. Ratios are all v/v %) was prepared in advanced and left at

60°C for at least 48 h. Once polymerized, samples were finally cut with an ultra-microtome (LKW) in 2 µm and 60-90 nm specimens, of which the ones of higher thickness (semi-thin slides) were left unstained (osmicated sections) or further stained with toluidine blue and used to observe skin morphology using LTM, whereas the other ones were deposited onto copper grids and stained with a uranyl acetate saturated solution (2 h) and then with a diluted solution of bismuth sub-nitrate (2 h) prior to be ready for TEM observation (JEOL JEM-100S operating at 80-100 kV). If otherwise indicated, sample preparation was conducted at room temperature.

## Results

Maghemite ( $\gamma\text{-Fe}_2\text{O}_3$ ) and iron (Fe) nanoparticle syntheses were easy and highly reproducible. Syntheses included a stabilization step to prevent irreversible particle aggregation upon aqueous dispersion. Stabilization was achieved by adsorbing tetramethylammonium hydroxide (TMAOH; a mild interaction) and sodium bis(2-ethylhexyl) sulfosuccinate (AOT; a strong interaction) counter-ions on maghemite and iron nanoparticle surface, respectively.

X-ray electron diffraction measurements revealed that TMAOH-stabilized maghemite nanoparticles were homogeneous in size and as small as  $6.94 \pm 0.86$  nm. These results were also confirmed by transmission electron microscope (TEM) visualizations (Fig. 23a). Nonetheless, and despite stabilization, TMAOH-maghemite nanoparticles were found as individual particles and arranged in reversible clusters (flocules) of variable sizes (up to 20 µm) upon dispersion in a basic aqueous medium, as confirmed by dynamic light scattering (DLS) measurements (Fig. 23c). It was also observed that flocculation increases by decreasing the pH of the dispersing medium, which indirectly confirmed that TMA ions interact weakly with maghemite nanoparticle surfaces. Dimensions of AOT-stabilized iron nanoparticles were obtained by TEM and DLS measurements. TEM showed that AOT-iron nanoparticle dispersions were rich in AOT and that contained particles of different sizes (Fig. 23b). However, 50% of observed nanoparticles have a diameter of  $4.86 \pm 1.32$  nm (Fig. 23b). DLS measurements revealed, in addition, the presence of larger flocules (80 nm average size) (Fig. 23d).

Both nanoparticles had superparamagnetic properties (REF) and oxidized to a certain extent (not calculated) when exposed to air, hence, their being referred to as 'superparamagnetic iron oxide nanoparticles' (SPIONs). SPION dimensions have been considered the most important parameter since permeation through the skin can occur through pilosebaceous pores (d: 10-70 µm), sweat

glands pores (d: 60-80  $\mu\text{m}$ ) and most commonly through the lipo-proteic matrix that cements stratum corneum (SC) dead corneocytes (inter-corneocyte distance of 75 nm in air-dried conditions; aqueous pores of c.a.  $28 \pm 13$  Å and repeating fluid lipophilic areas, whose dimensions have not been extrapolated yet, within the lipidic bilayers that measure c.a. 13 or 6 nm depending on lamellar organization of SC lipids. Comparing skin openings and SPION dimensions, it was hypothesized that SPIONs might be small enough to potentially penetrate into the skin. Though, the extent of permeation was considered to strongly depend on all the interactions that could occur between SPIONs and skin components and/or structures.

To avoid skin variability among species, permeation experiments were conducted ex-vivo using skin pieces of healthy human female donors that did not have a cutaneous-disease history. The age of donors appeared to not influence skin barrier properties since specific skin electrical resistivity calculated at time 0 were in accordance with normal skin values ( $12\text{-}120 \text{ k}\Omega\text{cm}^2$ , DC) and in the order of  $8.77 \pm 1.38$  and  $73.72 \pm 23.67 \text{ k}\Omega\text{cm}^2$  when values were respectively extrapolated from current measures at 1 kHz and 10 Hz. These frequencies were chosen to minimize capacitance contributions of viable epidermis on skin adsorption might have sequestered -OH ions excess in TMAOH-SPION dispersion limiting its destructive action on epithelia. In contrast, and perhaps due to AOT excess (Fig. 23b) and its surfactant properties, AOT-SPION dispersion produced a decrease of c.a.  $3.8 \text{ k}\Omega\text{cm}$  ( $p \leq 2 \times 10^{-6}$ ) already after 3 hours of skin contact. In this case, AOT-induced skin modification correlated with a decrease of  $28.27 \text{ k}\Omega\text{cm}^2$  resistivity values, whose modifications, if any, can be then considered as the reflection of SC barrier alteration. And indeed, few modifications in skin resistivity were observed at the end of permeation experiments (Figs 24A-B). These changes might be considered of small entity since, due to a wider resistivity SD at 10 Hz, they were generally visible only at 1 kHz. In particular, maghemite dispersion caused a decrease in skin resistivity of c.a.  $3 \text{ k}\Omega\text{cm}^2$  ( $p \leq 0.007$ ) after 12 hours of skin contact (Fig. 24A). This barrier perturbation is very mild when compared with the abrupt decrease (c.a.  $6.5 \text{ k}\Omega\text{cm}^2$  at 1 kHz with a  $p \leq 2.2 \times 10^{-6}$  and c.a.  $53.5 \text{ k}\Omega\text{cm}^2$  at 10 Hz with a  $p \leq 0.002$ ) in skin resistivity caused by TMAOH control solution (BI in Fig 24A), which was visible at both frequencies. This result was not expected since either TMAOH-maghemite dispersion and TMAOH control solution should have been at pH 12. It was then discovered that a slow adsorption of  $\text{CO}_2$  decreased the basicity of nanoparticle dispersion down to pH 7 without precipitating particle floccules (the physico-chemical reason is under investigation), which in turn explained the observed discrepancy in skin resistivity. It has been hypothesized that

CO<sub>2</sub> ( $p \leq 0.002$ ) in skin resistivity, which became visible, at 10 Hz, after 12 hours of contact. Due to AOT-SPION preparation method it was difficult to reproduce exactly its control solution, thus a PBS solution (W in Fig. 24B) was used instead. It should be said that a PBS solution was utilized in both experiments as a double-blank: no significant modifications of skin resistivity were observed in both experiments and frequencies when skin resistivity values of a PBS-treated specimen were compared to initial skin resistivity values (T-1 and T0) up to 12 hours of contact. Longer exposure to aqueous solutions has been, in fact, reported to modify SC architecture.

Skin resistivity measurements have been confirmed by skin morphology observations. In fact, light transmission microscope (LTM) visualization on toluidine blue-stained and osmicated 2  $\mu$ m-thick sections revealed that each skin piece had a good morphology, as assessed by the constant presence of 10-15 SC layers, a compact epidermis formed by a variable number (3-5 to 8-12) of cell planes, and a collagen and muscle filled dermis (Fig. 25A). No macroscopic modification of skin architecture was observed in PBS-exposed specimens indicating that protocols used from explant to fixation did not damage skin, at least macroscopically. In addition, a closer look at the structure of skin samples, which were put in contact with SPION dispersions and their control solutions, revealed few differences of small entity, which were in agreement with skin resistivity measurements. In particular, specimens exposed to TMAOH control solution showed a less densely-packed SC and a larger distance between epidermal cells as compared with PBS-exposed specimens (not shown). As previously observed for skin resistivity, these modifications did not occur on skin pieces treated with TMAOH-SPIONs (Fig 25B), confirming a correlation existing between skin morphology and its barrier. Modifications of skin architecture resembling those just described for TMAOH control solution were also suffered by specimens exposed to AOT-SPIONs (Fig. 25C). In this case, large gaps between epidermal cells were commonly observed. Even in this case, modifications seem to be in agreement with skin resistivity measurements.

Since skin resistivity measurements and skin morphology observations were in agreement, and since a decrease in skin resistivity is associated with better permeation of a molecule through the skin, these results suggested that AOT-SPIONs might permeate skin better than TMAOH-SPIONs. Quantification of iron content within the liquids that should have received permeating TMAOH- and AOT-SPIONs, measured via inductively coupled plasma-optical emission spectrometer (ICP-OES), produced inconclusive results. Nevertheless, microscopic examination of the skin surface and its



invaginations and the persistence of stain, despite multiple washes with PBS, contrasted the ICP-quantification results.

In order to determine whether SPIONs might have penetrated through the skin but only to a certain extent, a series of different microscope investigations on nanoparticle-treated skin-pieces were performed. Further LTM visualizations on hematoxylin-stained 10  $\mu\text{m}$ -thick sections showed that one or more brownish lines were always found on the surface and within SC layers. This observation was strengthened if skin pieces were preventively hydrated for 24 hours with PBS prior to start the permeation experiments, as shown in Fig. 25D. Since these colored areas were never found in controls, SPIONs have likely formed intercellular deposits within the SC structure. In addition, it was frequently observed a fine brownish granule accumulation in and between cells of stratum granulosum of epidermis when SPION-treated sections were observed at higher magnifications (Fig. 25E, but not shown in detail due to lost of resolution during image digitalization). Considering that melanin is located in the cytoplasm of epidermal basal cells (melanocytes), whose granule-containing extensions do not generally reach the stratum granulosum and are commonly phagocytosed by other epidermal cells it was hypothesized that the observed intercellular granulosity might be most likely due to SPION that succeeded reaching the epidermis. However, since even a magnification of 100X would not discriminate the real nature of that fine granulosity (i.e., melanin granules, keratohyalin granules of stratum granulosum, SPION deposits), sections were investigated at higher magnification.

Using high resolution scanning electron microscope (HR-SEM) equipped with an X-ray microanalyzer (energy dispersion spectrometry, EDS) SPION distribution within the thickness of 16  $\mu\text{m}$ -thick treated skins was revealed (Figs 26A-H). EDS-SEM showed that SPIONs diffused through the skin not homogeneously and formed, most of the time, roundish aggregates (Figs 26A-H). SPIONs were always found in SC and epidermis; basal layer of epidermis appeared to modulate further penetration since many SPION aggregates were found close to it. This observation may be explained by the presence of basal lamina, which is composed by a network of glycoproteins having the function of anchor epidermis to dermis. This network is also believed to act as a filter for large molecules (> 40,000 Da) and attempting to diffuse into the dermis. Hair infundibulum and normal skin invaginations and corrugations also appeared to be privileged places where SPION deposits were found (Figs 26A, C, B). In contrast, localization of SPIONs into the dermis was rare (Figs. 26H). Differences between TMAOH- and AOT-SPIONs were found to be restricted to the larger

number of AOT-SPION deposits found within the skin, a finding consistent with the skin resistivity measurements.

For the first time, rigid nanoparticles of roughly 5 and 7 nm, were shown to penetrate the skin,  
5 without any necessity for applying an external electromagnetic field, and the particles were found to  
penetrate deeply, even at times, reaching the dermis.

While certain features of the invention have been illustrated and described herein, many  
modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the  
10 art. It is, therefore, to be understood that the appended claims are intended to cover all such  
modifications and changes as fall within the true spirit of the invention.

## CLAIMS

What is claimed is:

1. A topical delivery system for skin retention of a lipophilic agent in a subject, said system comprising:
  - a. a saturating agent; and
  - b. a lipophilic agent in a vehicle, wherein said vehicle comprises a polar solvent, a surfactant, an organic solvent or a combination thereof;
2. The delivery system of claim 1, wherein said saturating agent is a polar solvent, an organic solvent or a surfactant.
3. The delivery system of claim 1, wherein said saturating agent has a molecular weight of up to 1450 Da.
4. The delivery system of claim 1, wherein said vehicle has a molecular weight of up to 1450 Da.
5. The delivery system of claim 1, wherein said organic solvent is isopropyl myristate.
6. The delivery system of claim 1, wherein said surfactant is a Tween, a Span or 1,2-octanediol.
7. The delivery system of claim 1, wherein said polar solvent is water.
8. The delivery system of claim 1, wherein said saturating agent is a surfactant and said vehicle is water.
9. The delivery system of claim 1, wherein said saturating agent is an organic solvent and said vehicle is water.
10. The delivery system of claim 1, wherein said saturating agent and said vehicle is water.
11. The delivery system of claim 1, wherein said saturating agent is a surfactant and said vehicle is a surfactant.
12. The delivery system of claim 11, wherein said saturating agent is a Span or a Tween and said vehicle is 1,2-octanediol.
13. The delivery system of claim 11, wherein said saturating agent is 1,2-octanediol and said vehicle is a Span or Tween.
14. The delivery system of claim 1, wherein said saturating agent is an organic solvent and said vehicle is a surfactant.
15. The delivery system of claim 1, wherein said saturating agent is a surfactant and said vehicle is an organic solvent.
16. The delivery system of claim 1, wherein said lipophilic agent is retinoic acid.

17. The delivery system of claim 1, wherein said system is formulated for delivery from a patch.

18. A topical delivery system for lipophilic agent permeation of the skin, said system comprising:

a. a saturating agent; and

5 b. a lipophilic agent in solution in an organic solvent or a surfactant;

19. The delivery system of claim 18, wherein said saturating agent is a polar solvent, an organic solvent or a surfactant.

20. The delivery system of claim 18, wherein said saturating agent has a molecular weight of at least 18 Da.

10 21. The delivery system of claim 18, wherein said vehicle has a molecular weight of at least 18 Da.

22. The delivery system of claim 18, wherein said organic solvent is isopropyl myristate.

23. The delivery system of claim 18, wherein said surfactant is a Tween, a Span or 1,2-octanediol.

15 24. The delivery system of claim 18, wherein said polar solvent is water.

25. The delivery system of claim 18, wherein said saturating agent is a water or Tween and said vehicle is isopropyl myristate.

26. The delivery system of claim 18, wherein said saturating agent is isopropyl myristate or Span and said vehicle is Tween.

20 27. The delivery system of claim 18, wherein said saturating agent is Tween and said vehicle is Span.

28. The delivery system of claim 18, wherein said lipophilic agent is retinoic acid.

29. The delivery system of claim 18, wherein said system is formulated for delivery from a patch.

25 30. A topical delivery system, for retention of a hydrophilic agent, said system comprising:

a. a saturating agent; and

b. a hydrophilic drug in solution in a polar solvent, a surfactant or an organic solvent;

31. The delivery system of claim 30, wherein said saturating agent is a polar solvent, an organic solvent or a surfactant.

30 32. The delivery system of claim 30, wherein said saturating agent has a molecular weight of up to 1450 Da.

33. The delivery system of claim 30, wherein said vehicle has a molecular weight of up to 1450 Da.

34. The delivery system of claim 30, wherein said organic solvent is isopropyl myristate.

35. The delivery system of claim 30, wherein said surfactant is a Tween, a Span or 1,2-octanediol.

36. The delivery system of claim 30, wherein said polar solvent is water.

37. The delivery system of claim 30, wherein said saturating agent is an organic solvent, a surfactant, or a polar solvent, and said vehicle is water.

38. The delivery system of claim 30, wherein said saturating agent is an organic solvent and said vehicle is a surfactant.

39. The delivery system of claim 30, wherein said saturating agent is a surfactant and said vehicle is a surfactant.

40. The delivery system of claim 39, wherein said saturating agent is a Tween or 1, 2-octanediol and said vehicle is a Span.

41. The delivery system of claim 39, wherein said saturating agent is a Tween or a Span and said vehicle is 1, 2-octanediol.

42. The delivery system of claim 30, wherein said saturating agent is a surfactant and said vehicle is an organic solvent.

43. The delivery system of claim 30, wherein said saturating agent is an organic solvent and said vehicle is a surfactant.

44. The delivery system of claim 30, wherein said hydrophilic agent is mannitol.

45. The delivery system of claim 30, wherein said system is formulated for delivery from a patch.

46. A topical delivery system for hydrophilic agent permeation of the skin, said system comprising:

- a. a saturating agent; and
- b. a hydrophilic agent in solution in an organic solvent or a surfactant;

47. The delivery system of claim 46, wherein said saturating agent is a polar solvent, an organic solvent or a surfactant.

48. The delivery system of claim 46, wherein said saturating agent has a molecular weight of at least 18 Da.

49. The delivery system of claim 46, wherein said vehicle has a molecular weight of at least 18 Da.

50. The delivery system of claim 46, wherein said organic solvent is isopropyl myristate.

51. The delivery system of claim 46, wherein said surfactant is a Tween, a Span or 1,2-octanediol.

52. The delivery system of claim 46, wherein said polar solvent is water.

53. The delivery system of claim 46, wherein said saturating agent is a water or Tween and said vehicle is isopropyl myristate.

54. The delivery system of claim 46, wherein said saturating agent is isopropyl myristate or Span and said vehicle is Tween.

55. The delivery system of claim 46, wherein said saturating agent is Tween and said vehicle is Span.

56. The delivery system of claim 46, wherein said hydrophilic agent is mannitol.

57. The delivery system of claim 46, wherein said system is formulated for delivery from a patch.

58. A method of dermal or transdermal drug delivery in a subject, said method comprising:

- a. Contacting a skin surface of said subject with a saturating agent; and
- b. Contacting said skin surface with a drug in a vehicle, wherein said vehicle comprises a polar solvent, a surfactant, an organic solvent or a combination thereof;

whereby said drug is retained within, or permeates a skin in said subject, thereby being a method of dermal or transdermal drug delivery.

59. The method of claim 58, wherein said saturating agent is a polar solvent, an organic solvent or a surfactant.

60. The method of claim 58, wherein said organic solvent is isopropyl myristate.

61. The drug delivery system of claim 58, wherein said surfactant is a Tween, a Span or 1,2-octanediol.

62. The drug delivery system of claim 58, wherein said polar solvent is water.

63. The method of claim 58, wherein said drug is retained within said skin.

64. The method of claim 63, wherein said saturating agent has a molecular weight of up to 1450 Da.

65. The method of claim 63, wherein said vehicle has a molecular weight of up to 1450 Da.

66. The method of claim 63, wherein said drug is lipophilic.

67. The method of claim 63, wherein said saturating agent is a surfactant and said vehicle is water.

68. The method of claim 63, wherein said saturating agent is an organic solvent and said vehicle is water.

69. The method of claim 63, wherein said saturating agent and said vehicle is water.

70. The method of claim 63, wherein said saturating agent is a surfactant and said vehicle is a surfactant.

71. The method of claim 70, wherein said saturating agent is a Span or a Tween and said vehicle is 1,2-octanediol.

5 72. The method of claim 70, wherein said saturating agent is 1,2-octanediol and said vehicle is a Span or Tween.

73. The method of claim 63, wherein said saturating agent is an organic solvent and said vehicle is a surfactant.

10 74. The method of claim 63, wherein said saturating agent is a surfactant and said vehicle is an organic solvent.

75. The method of claim 63, wherein said lipophilic drug is retinoic acid.

76. The method of claim 63, wherein said drug is hydrophilic.

77. The method of claim 76, wherein said saturating agent is an organic solvent, a surfactant, or a polar solvent, and said vehicle is water.

15 78. The method of claim 76, wherein said saturating agent is an organic solvent and said vehicle is a surfactant.

79. The method of claim 76, wherein said saturating agent is a surfactant and said vehicle is a surfactant.

20 80. The method of claim 79, wherein said saturating agent is a Tween or 1, 2-octanediol and said vehicle is a Span.

81. The method of claim 79, wherein said saturating agent is a Tween or a Span and said vehicle is 1, 2-octanediol.

82. The method of claim 76, wherein said saturating agent is a surfactant and said vehicle is an organic solvent.

25 83. The method of claim 76, wherein said saturating agent is an organic solvent and said vehicle is a surfactant.

84. The method of claim 76, wherein said hydrophilic drug is mannitol.

85. The method of claim 58, wherein said drug permeates said skin.

30 86. The method of claim 85, wherein said saturating agent has a molecular weight of at least 18 Da.

87. The method of claim 85, wherein said vehicle has a molecular weight of at least 18 Da .

88. The method of claim 85, wherein said drug is lipophilic.

89. The method of claim 88, wherein said organic solvent is isopropyl myristate.

90. The method of claim 88, wherein said surfactant is a Tween, a Span or 1,2-octanediol.

91. The method of claim 88, wherein said polar solvent is water.

92. The method of claim 88, wherein said saturating agent is a water or Tween and said vehicle is isopropyl myristate.

93. The method of claim 88, wherein said saturating agent is isopropyl myristate or Span and said vehicle is Tween.

94. The method of claim 88, wherein said saturating agent is Tween and said vehicle is Span.

95. The method of claim 85, wherein said drug is hydrophilic.

96. The method of claim 95, wherein said saturating agent is a water or Tween and said vehicle is isopropyl myristate.

97. The method of claim 95, wherein said saturating agent is isopropyl myristate or Span and said vehicle is Tween.

98. The method of claim 95, wherein said saturating agent is Tween and said vehicle is Span.

99. The method of claim 58, wherein said drug treats a condition of the skin, nails, scalp, hands, feet, or combination thereof in said subject.

100. The method of claim 58, wherein said drug treats infection, inflammation, eczema, dermatitis, keratosis, urticaria, allergy, acnee, folliculitis, furuncles, psoriasis, rosacea, pityriasis, cancer, precancerous lesions, muscular pain, arthritis, heart disease, osteoporosis, osteopetrosis, burns, non-healing wounds, scars, skin ulcers, hyperhydrosis, ichthyosis, lupus of the skin, sun damage, vitiligo, or a combination thereof, in said subject.

101. The method of claim 58, wherein said drug is an analgesic, an anesthetic, an antioxidant, a growth factor, a hormone, an extracellular matrix component, or a combination thereof.

102. The method of claim 58, wherein said drug is formulated as a microemulsion.

103. The method of claim 58, wherein said saturating agent and said vehicle comprising a drug is formulated for delivery from a patch.



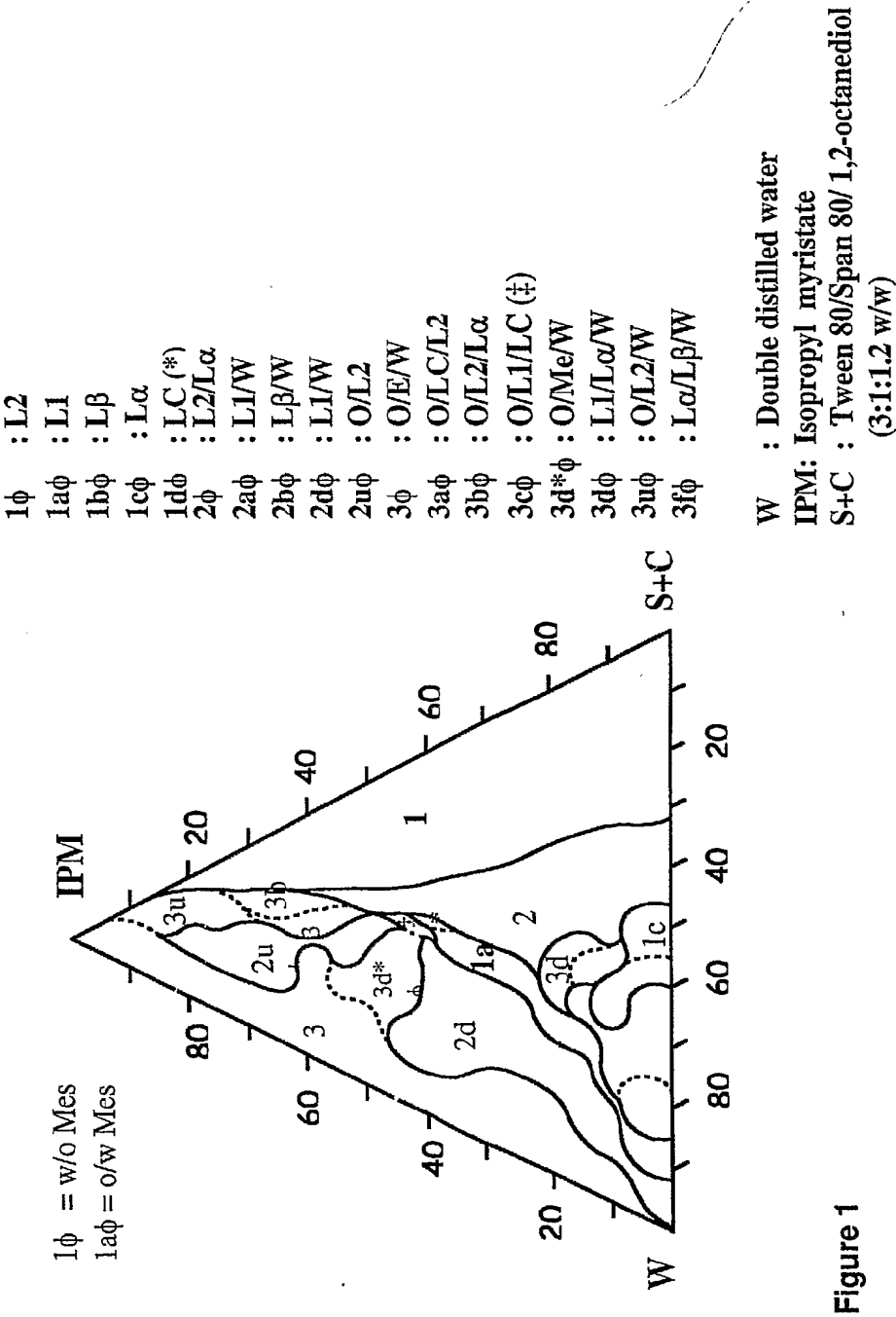


Figure 1

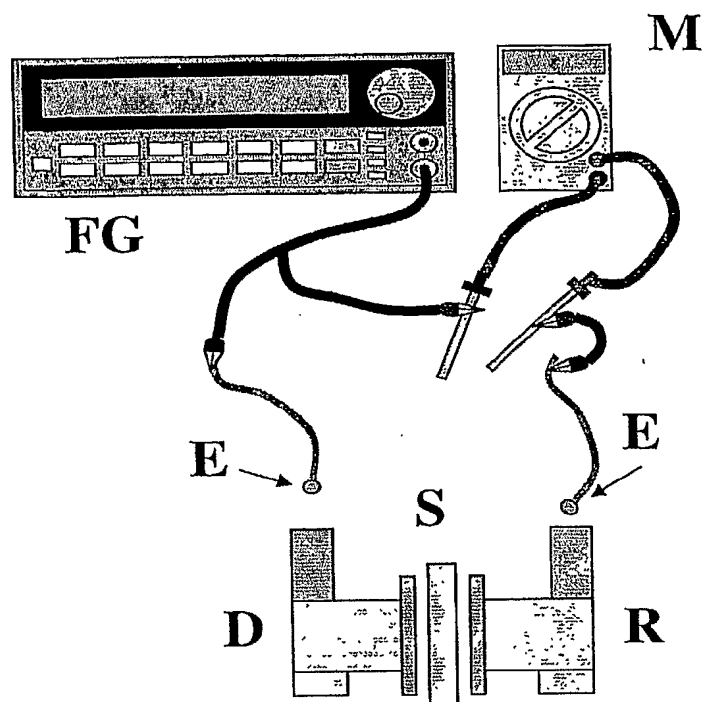
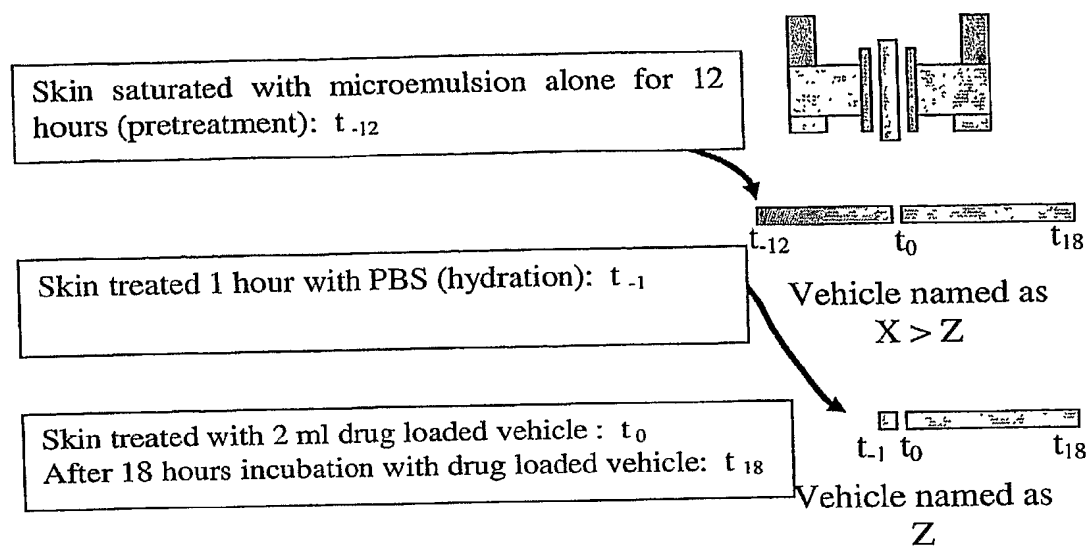


Figure 2

**Figure 2B**

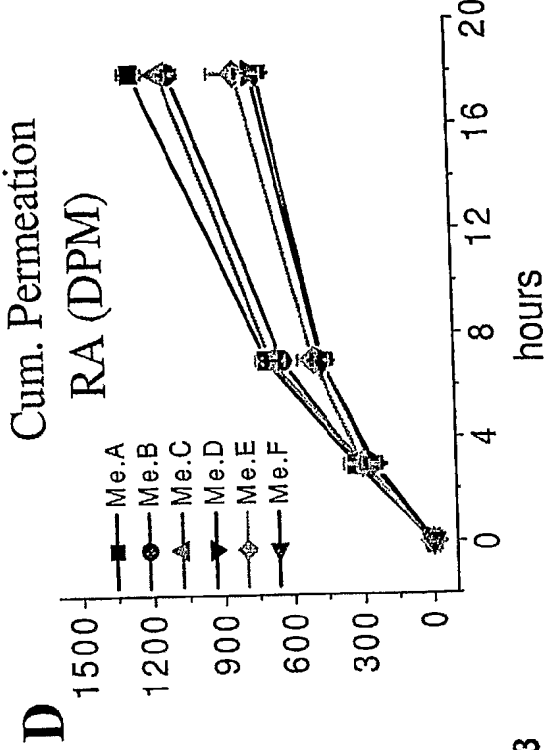
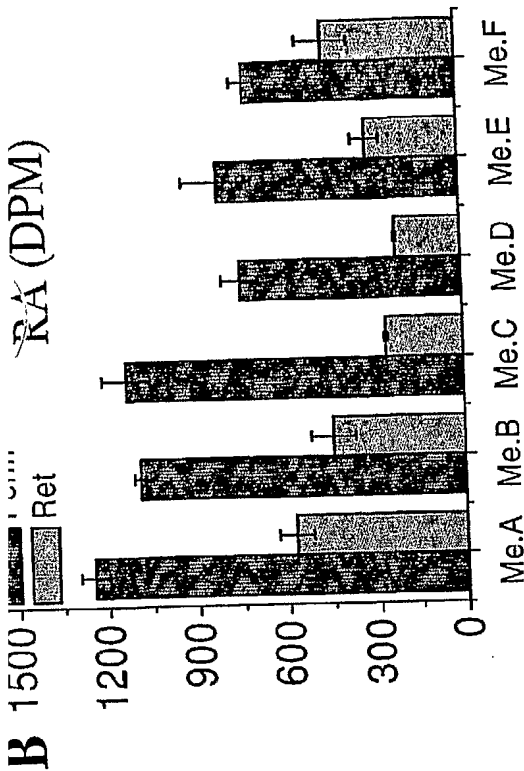
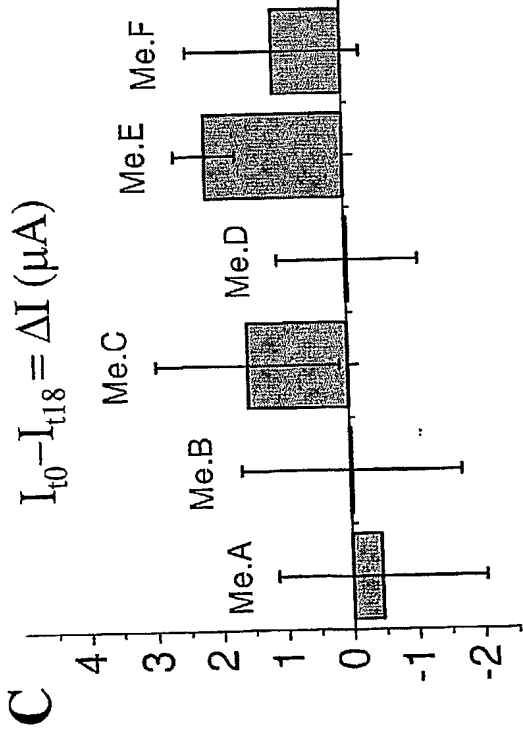
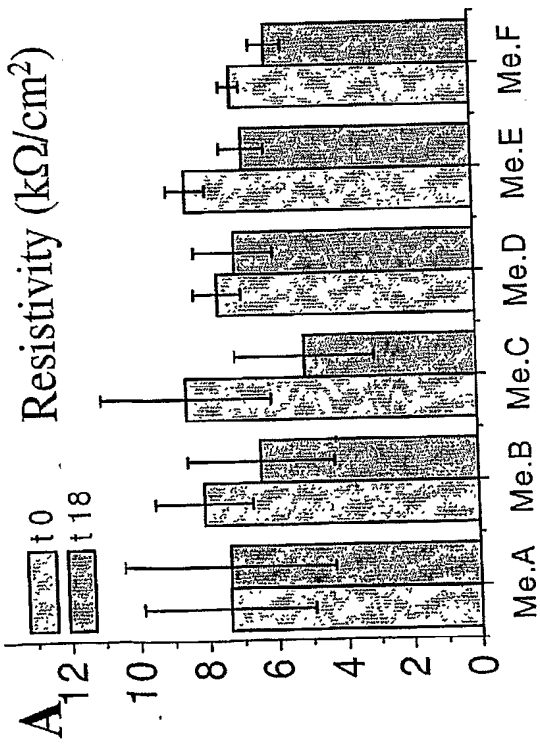


Figure 3

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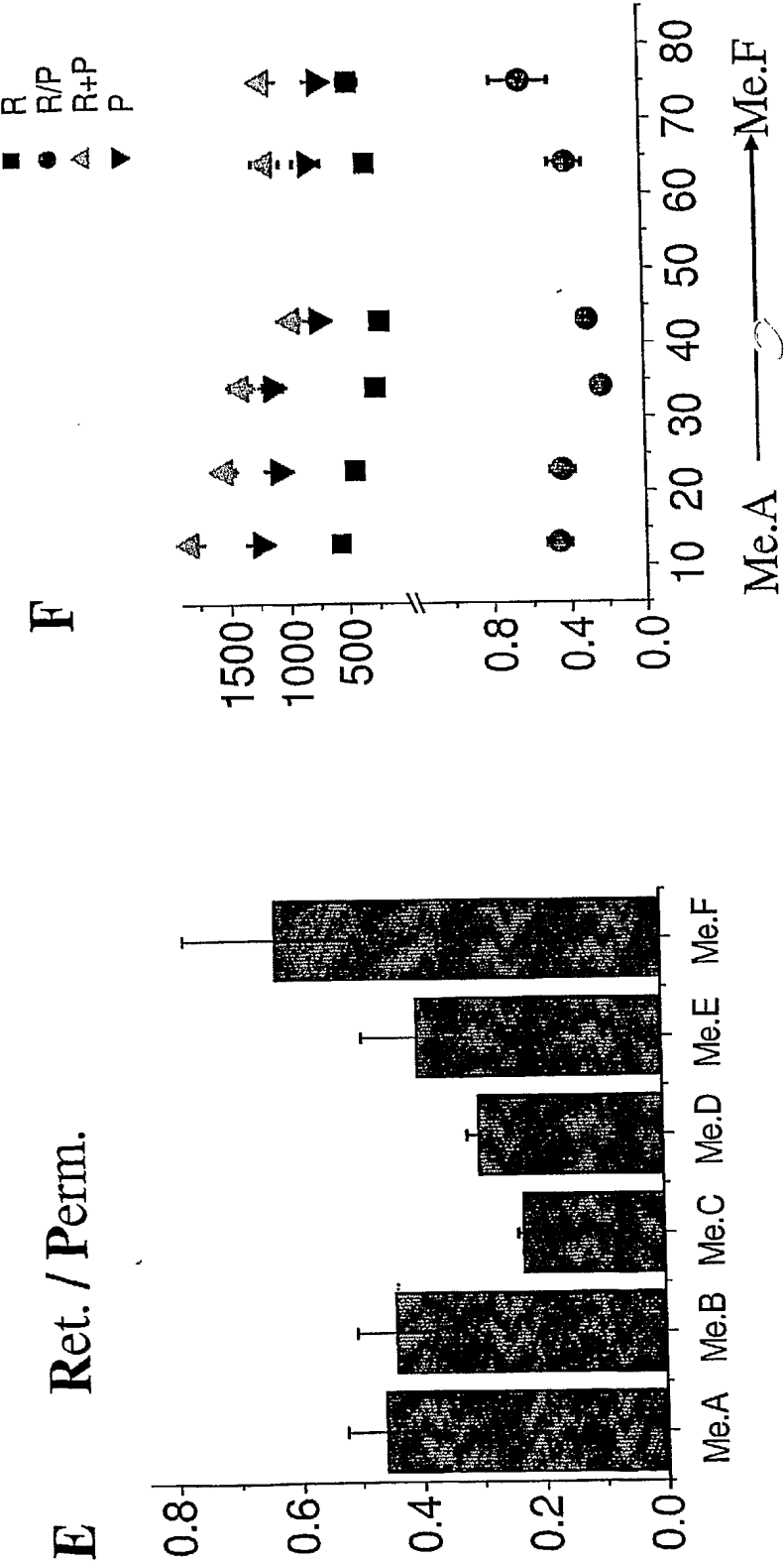


Figure 3

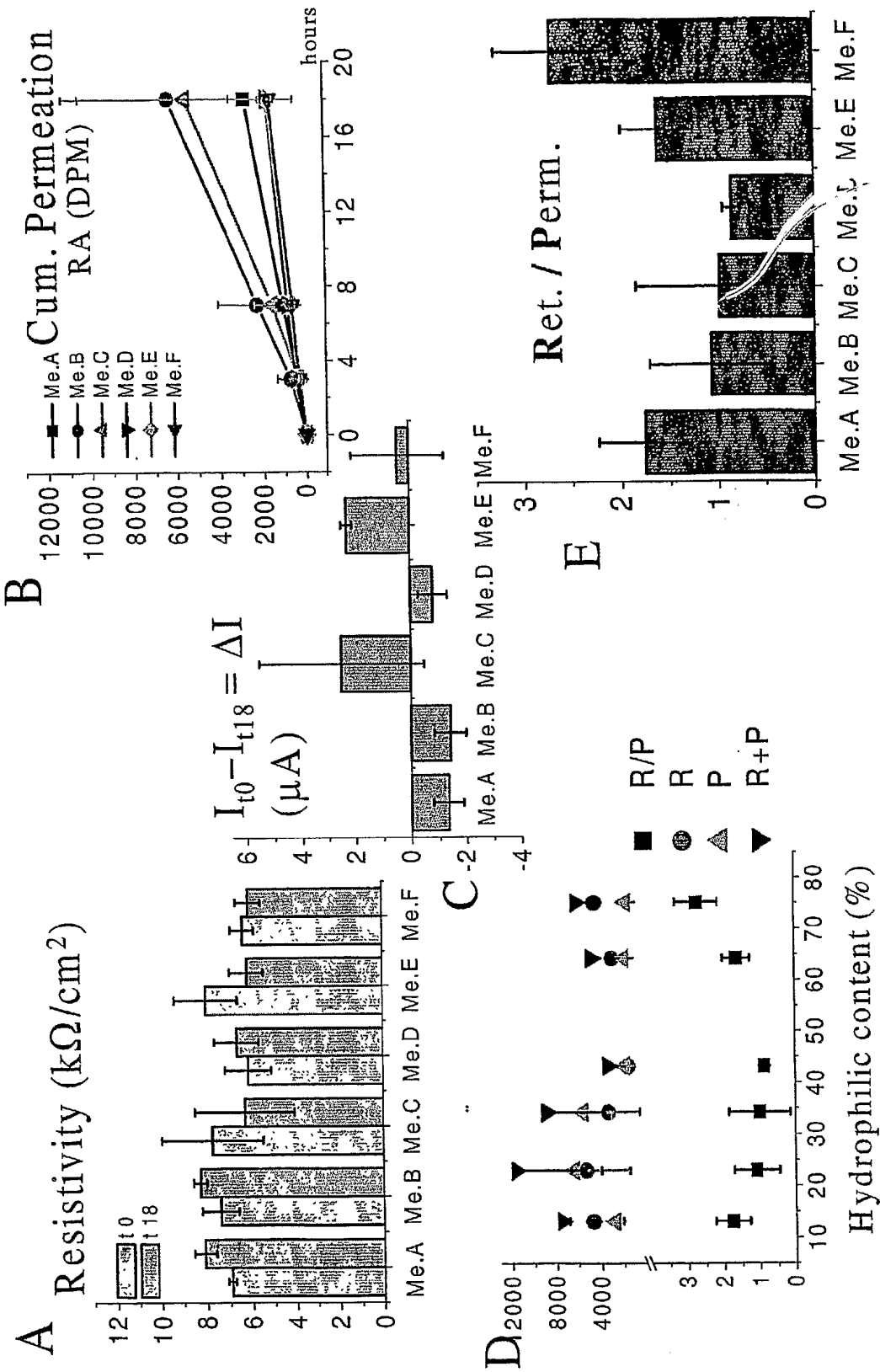


Figure 4

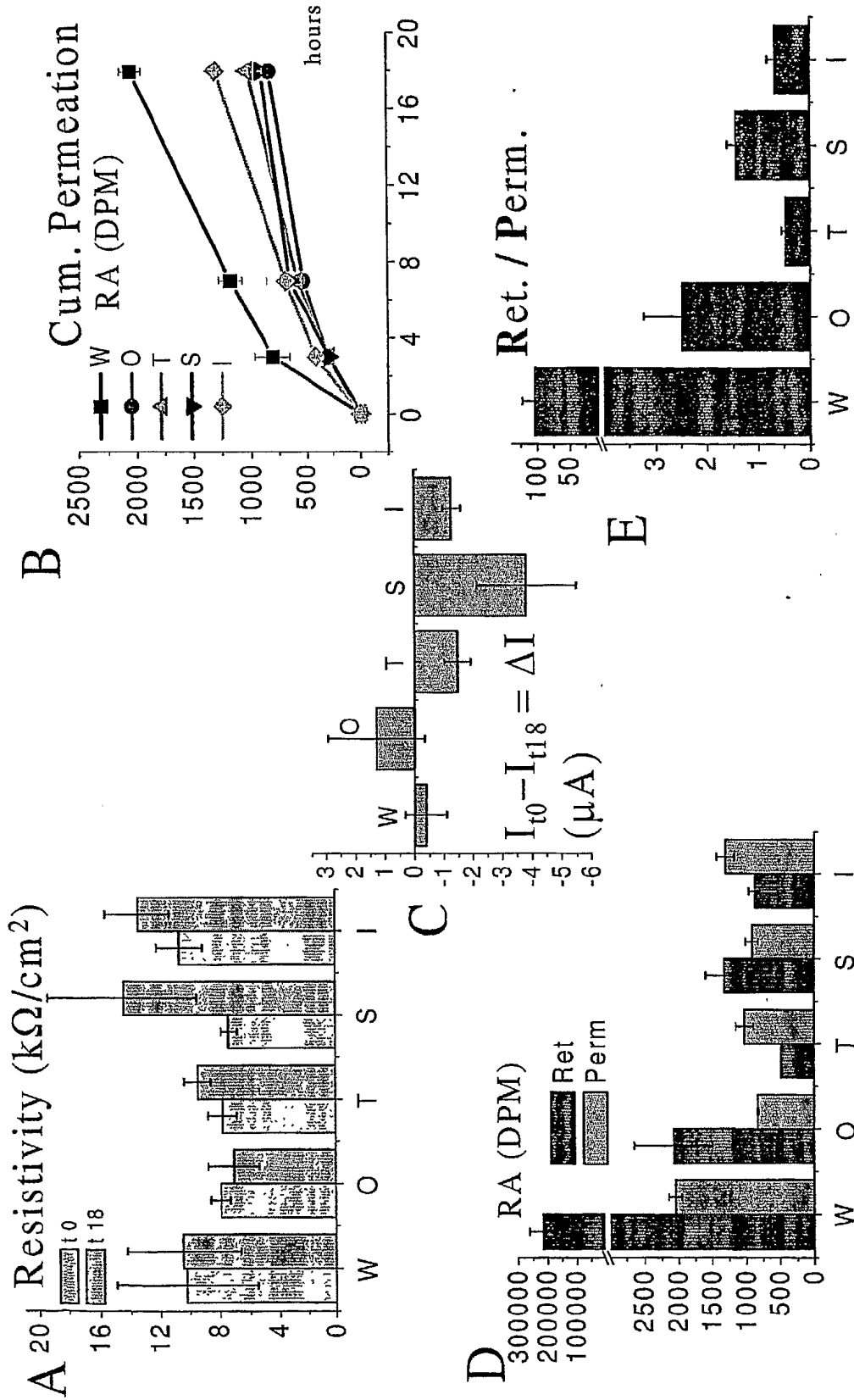
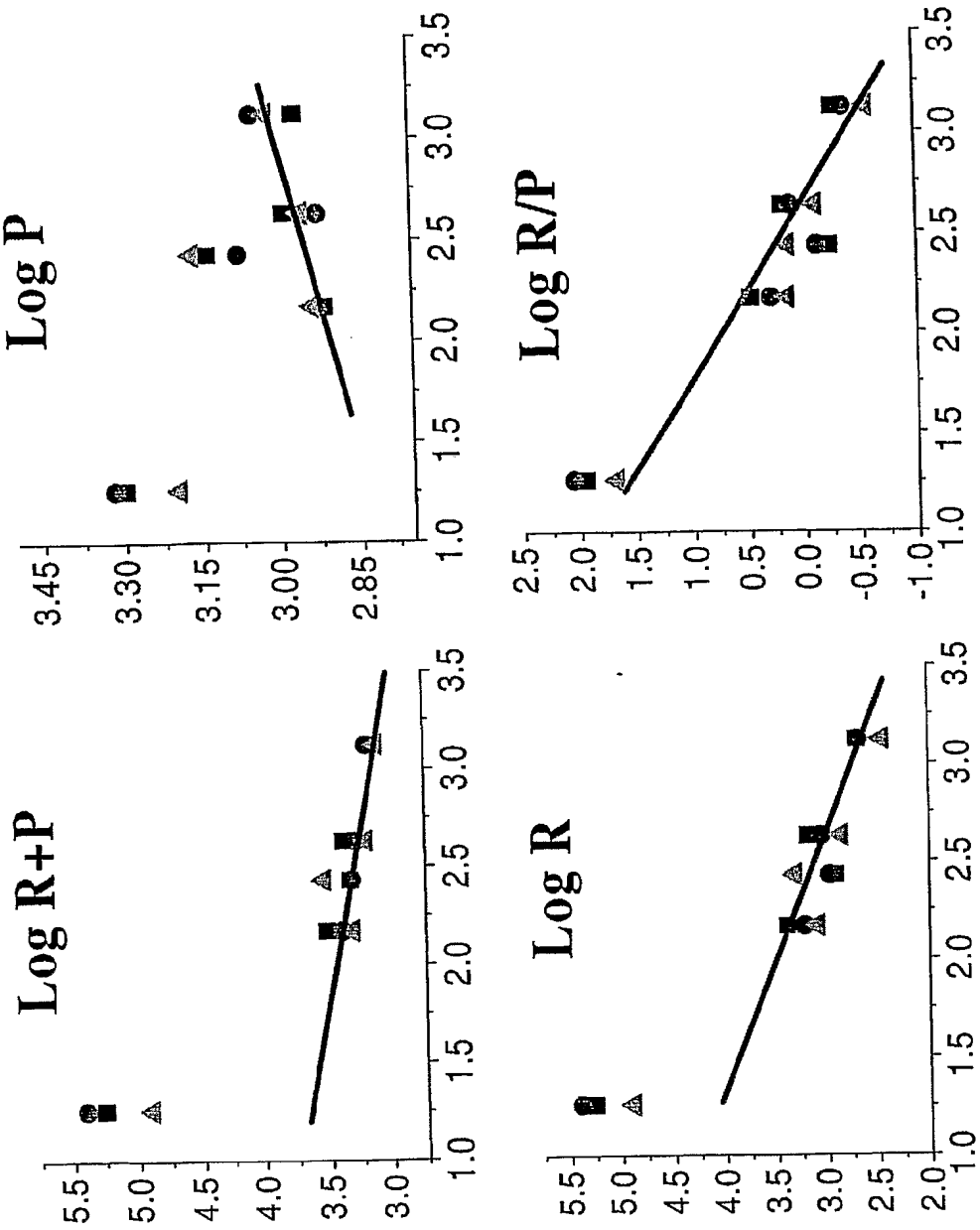


Figure 5



| Log MW = x |      |
|------------|------|
| W          | 1.26 |
| O          | 2.17 |
| I          | 2.43 |
| S          | 2.63 |
| T          | 3.12 |

Figure 6



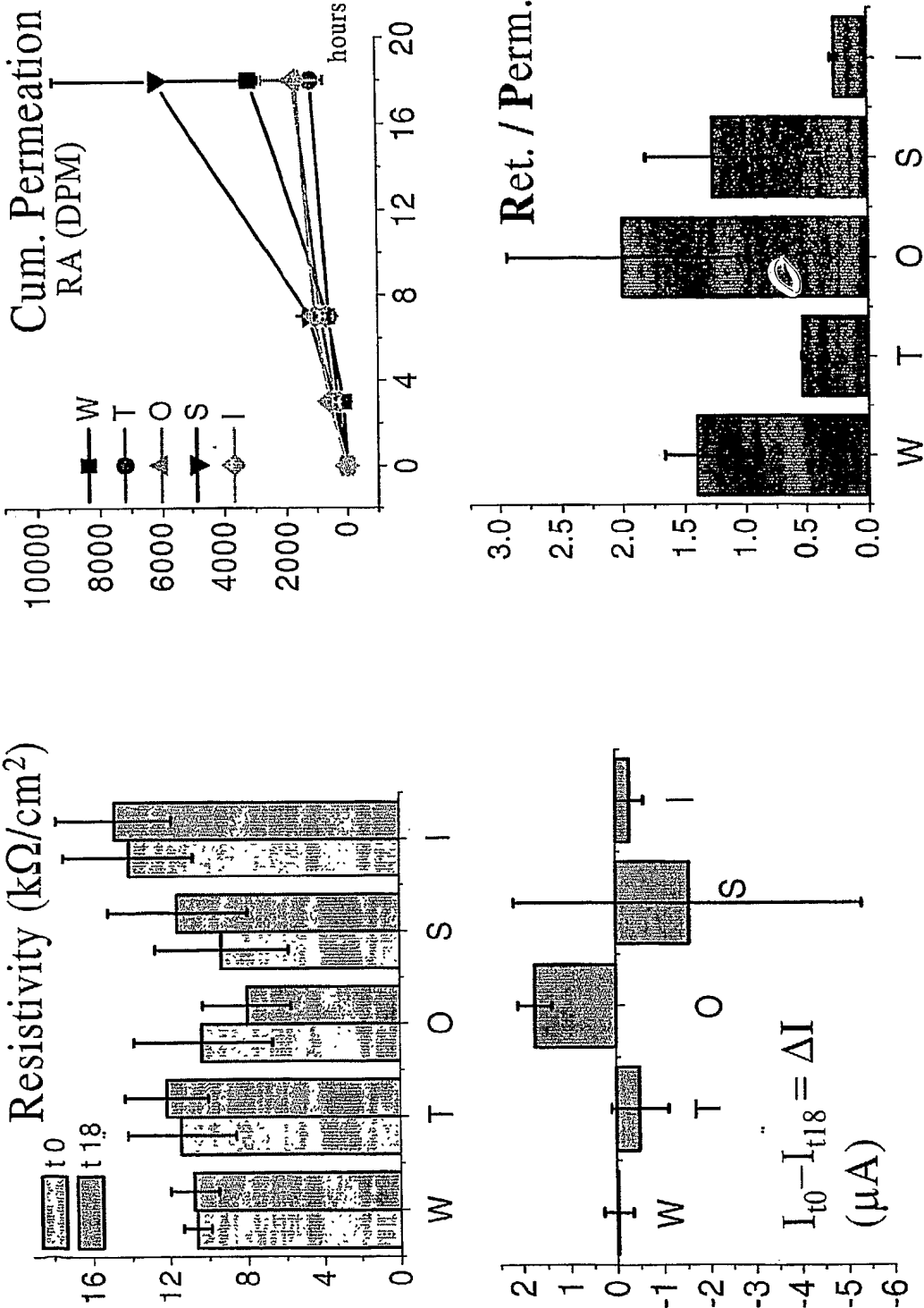


Figure 7

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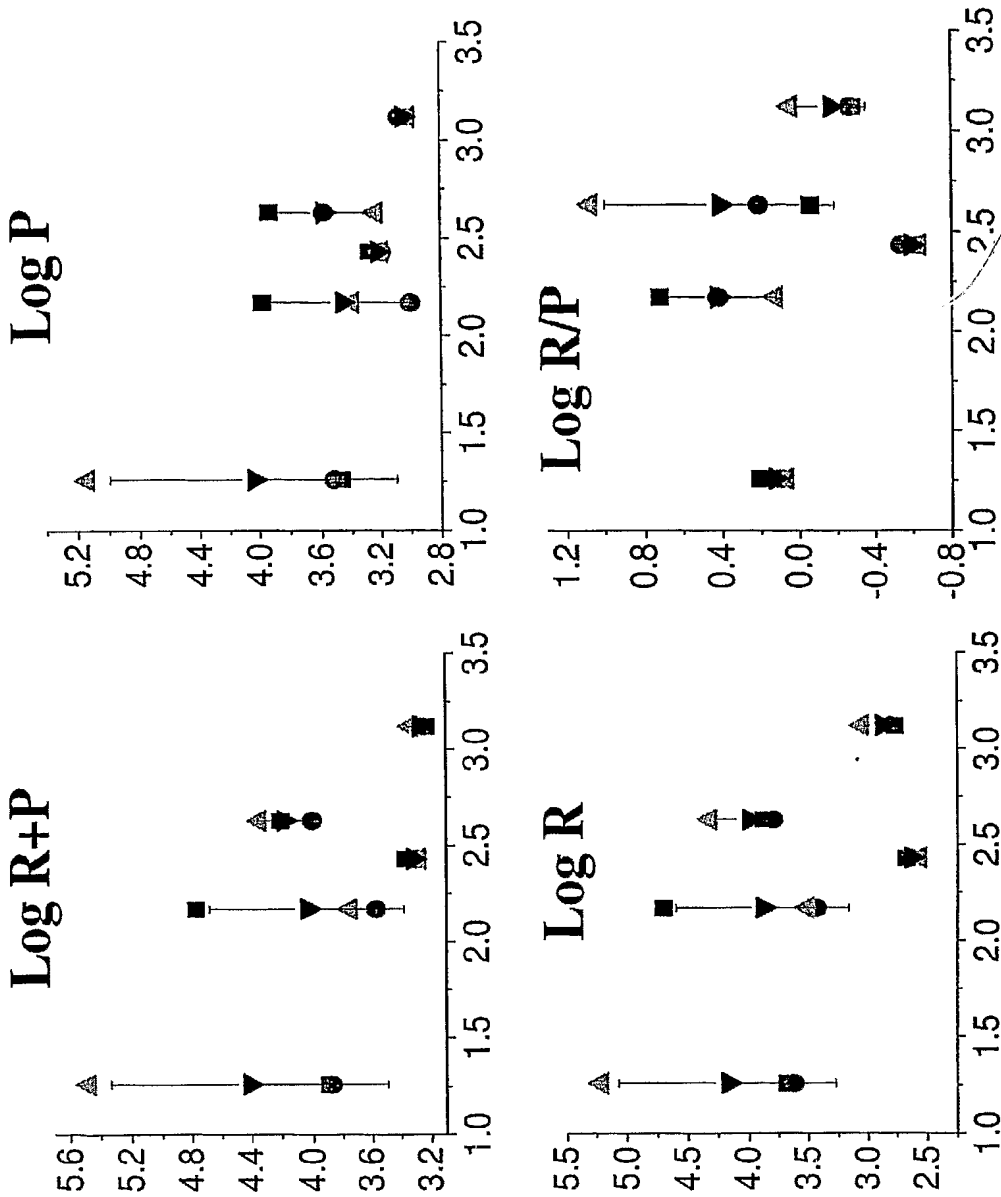


Figure 8

10/28

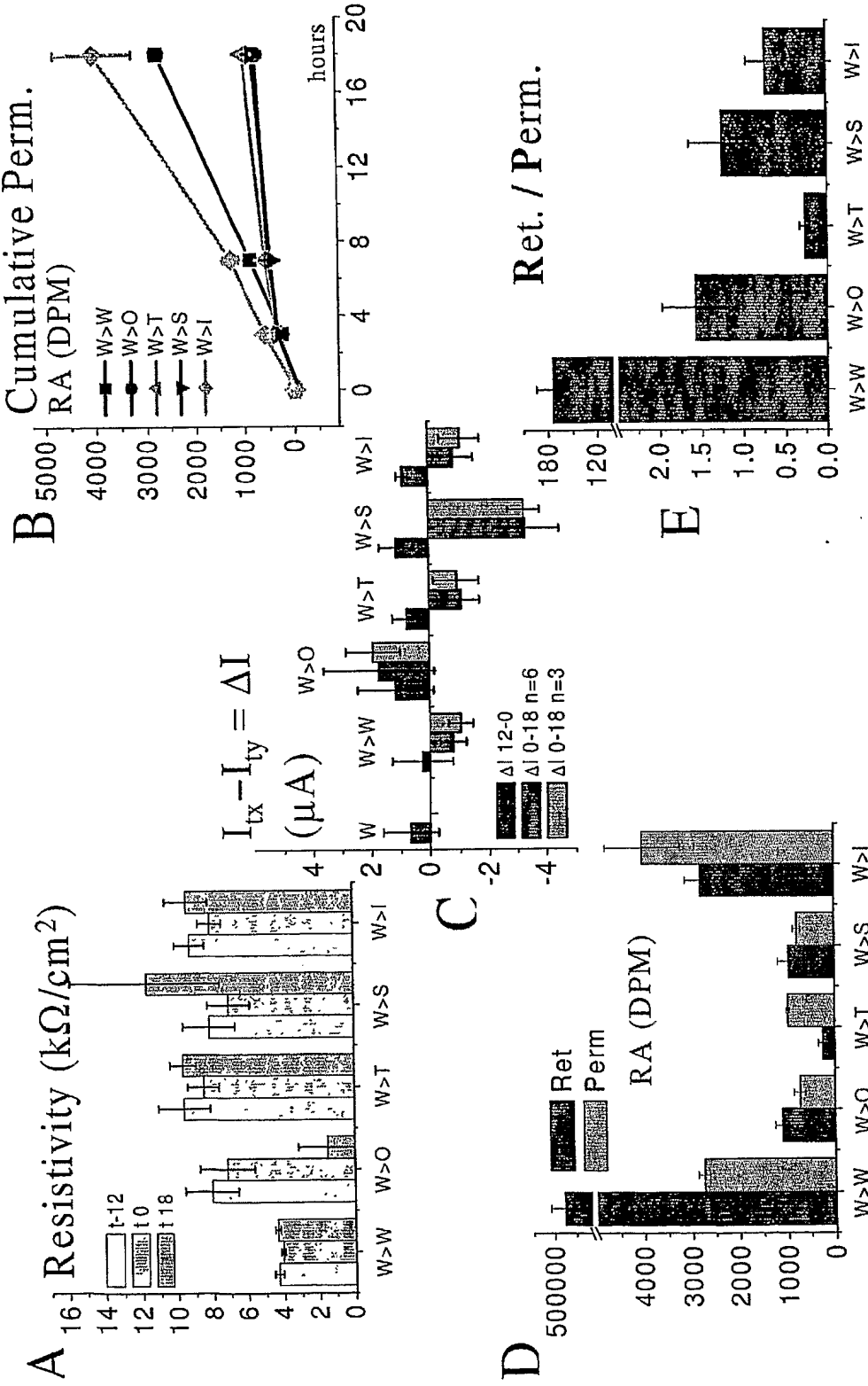


Figure 9

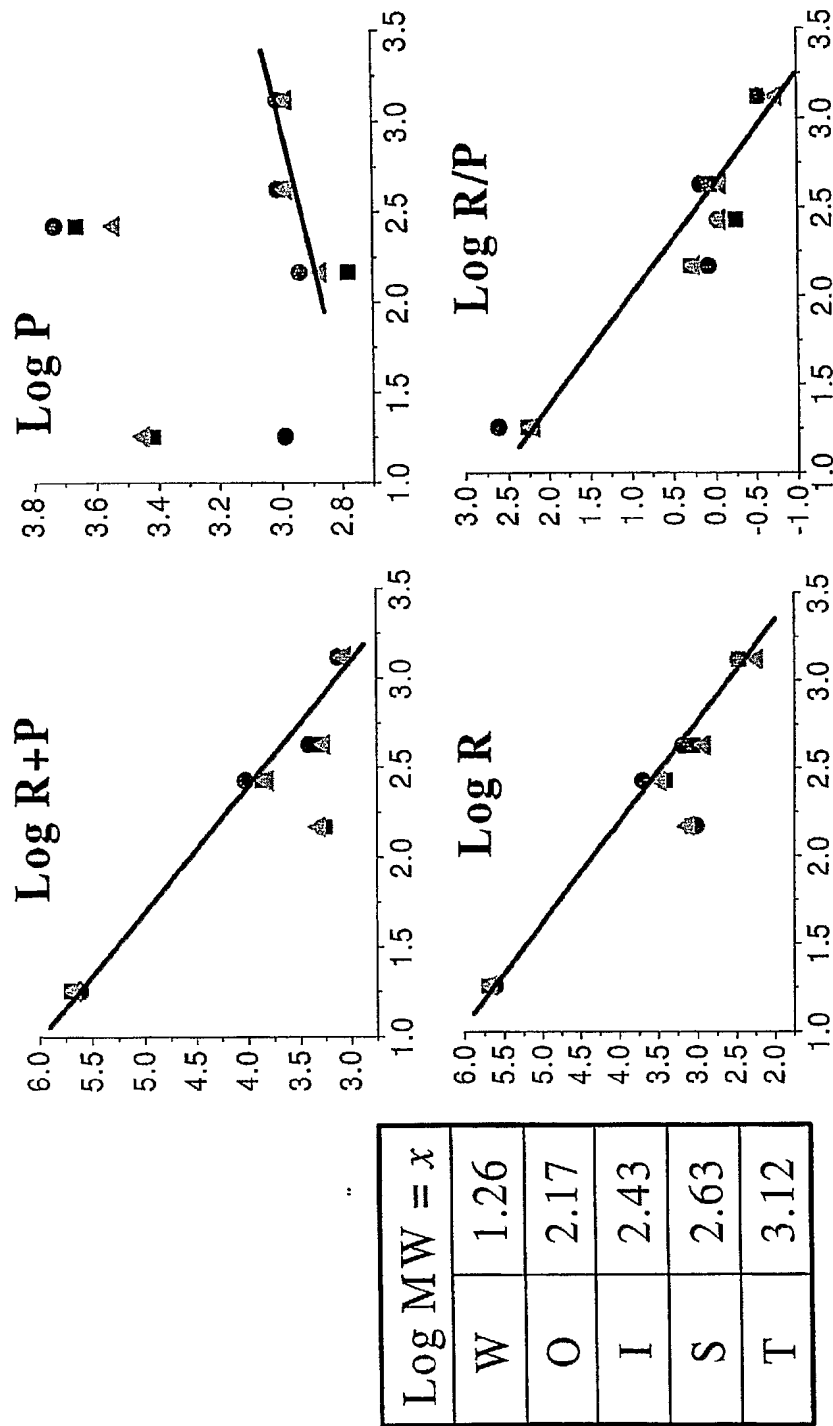


Figure 10

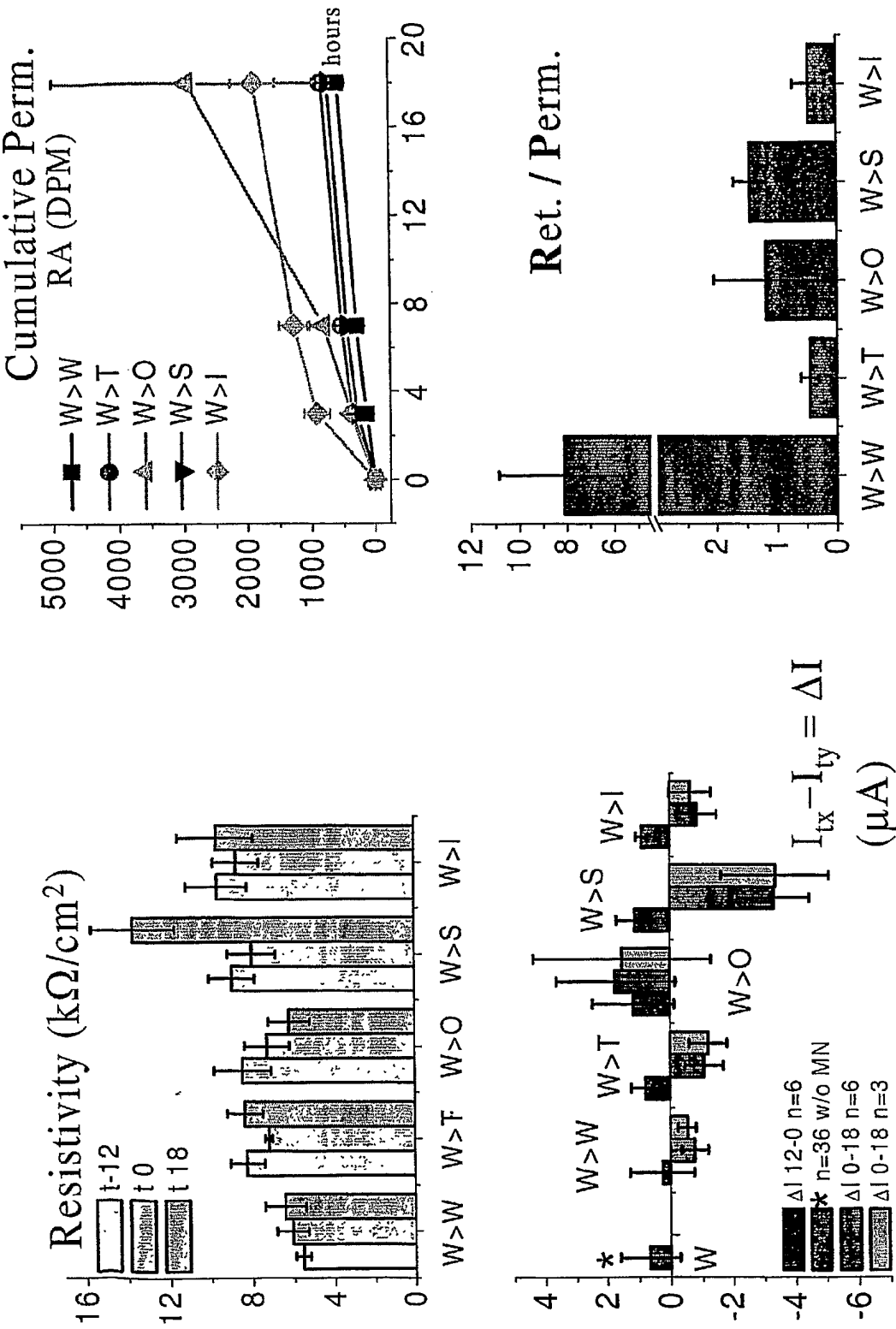


Figure 11

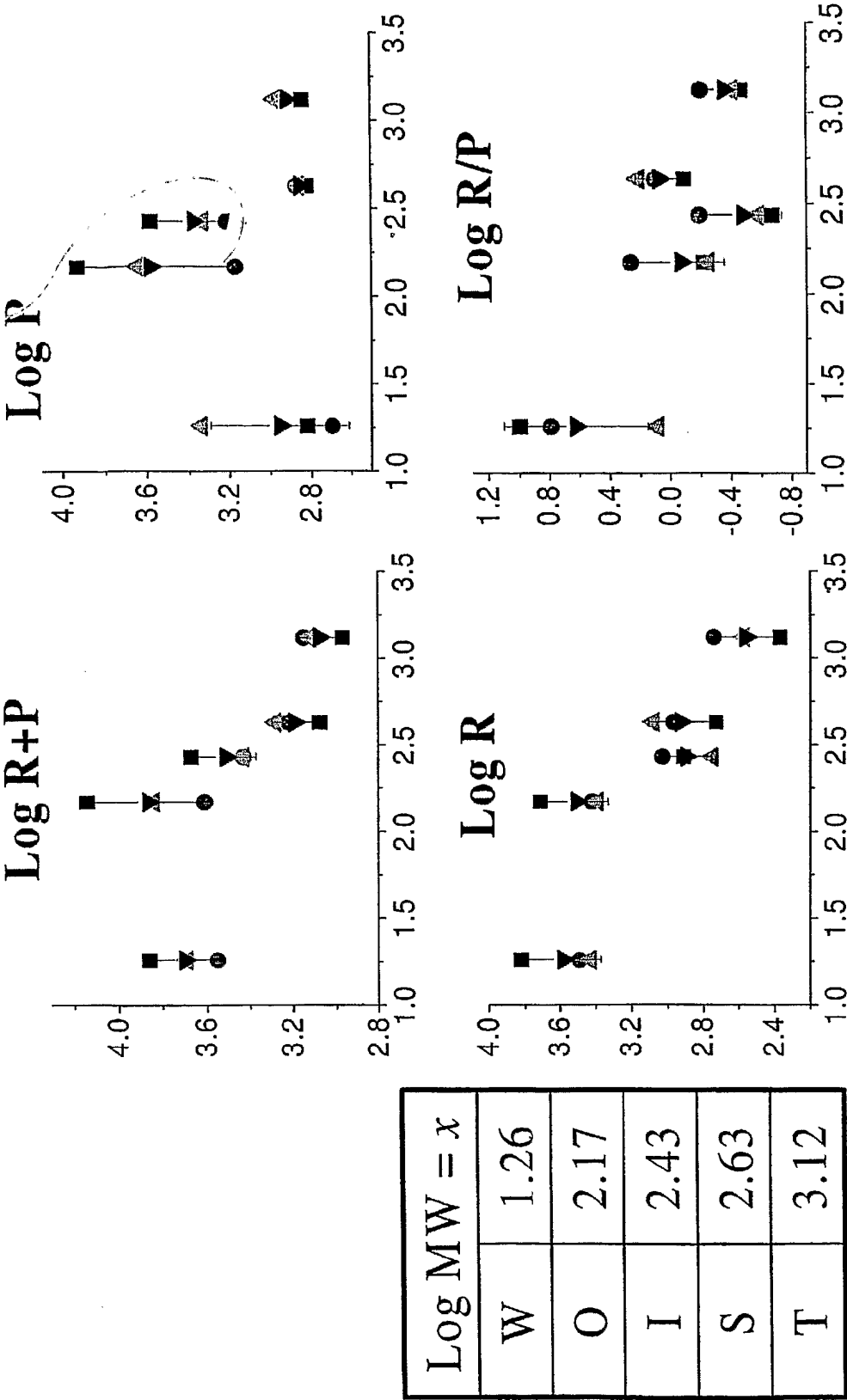


Figure 12

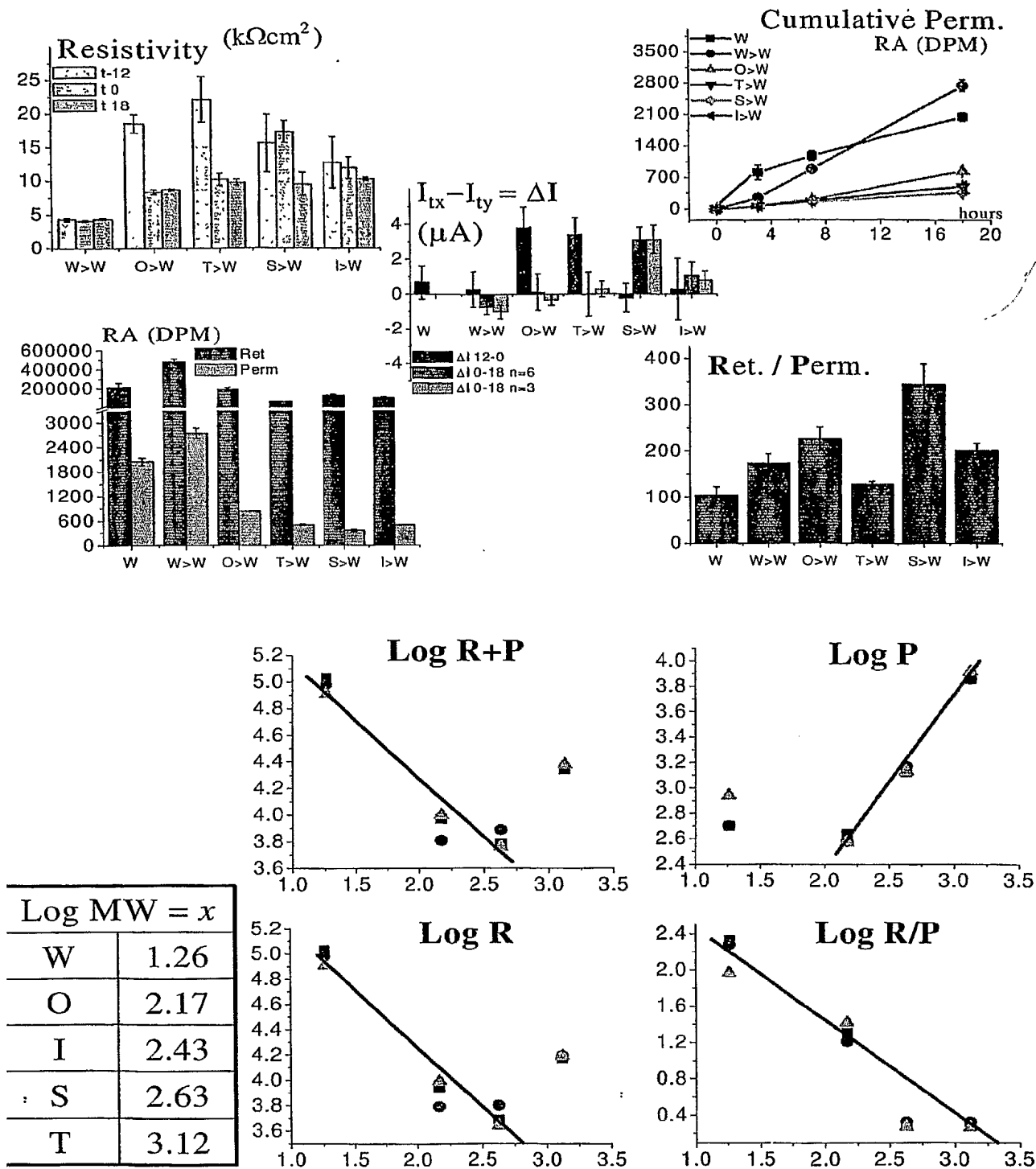


Figure 13

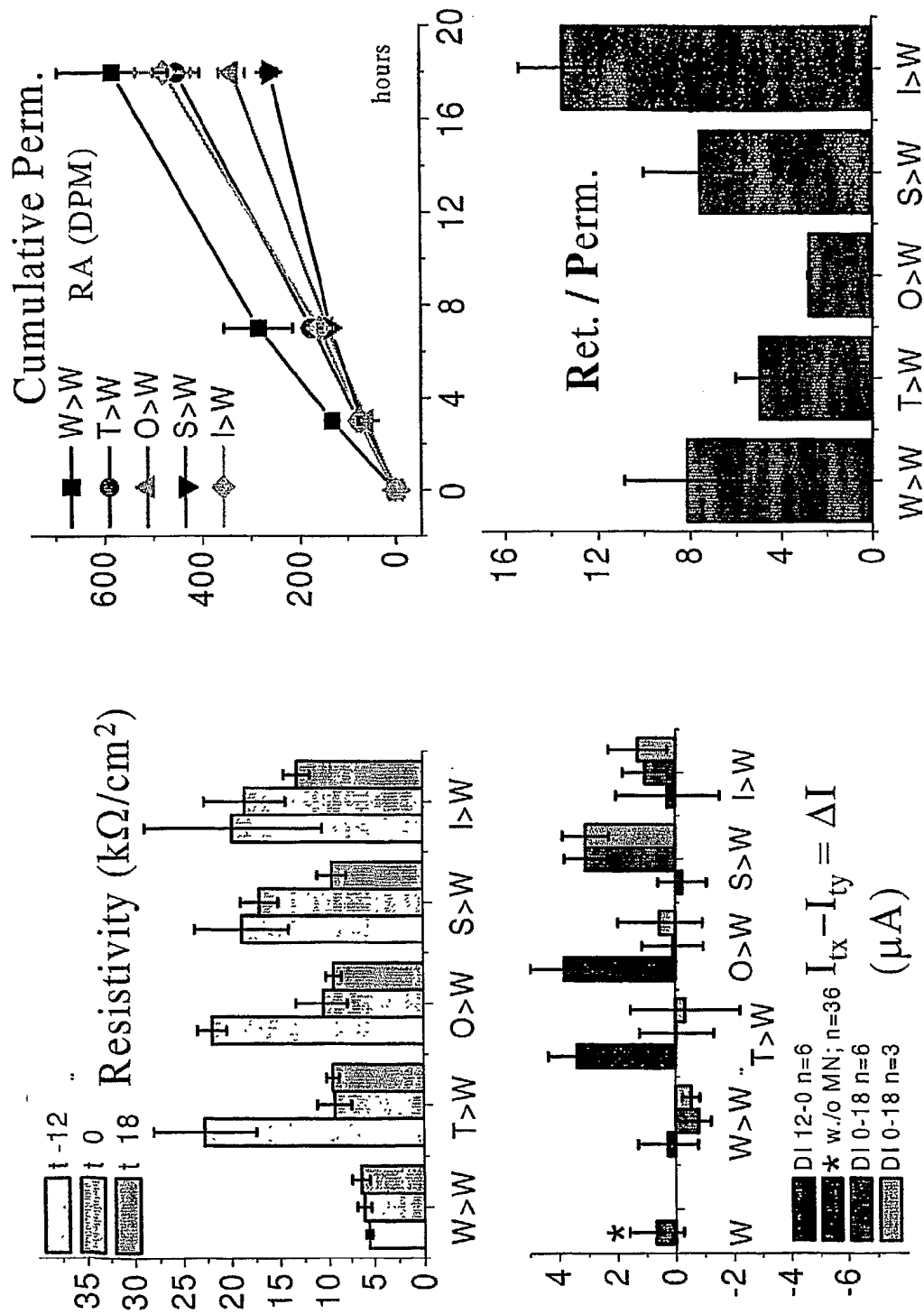


Figure 14



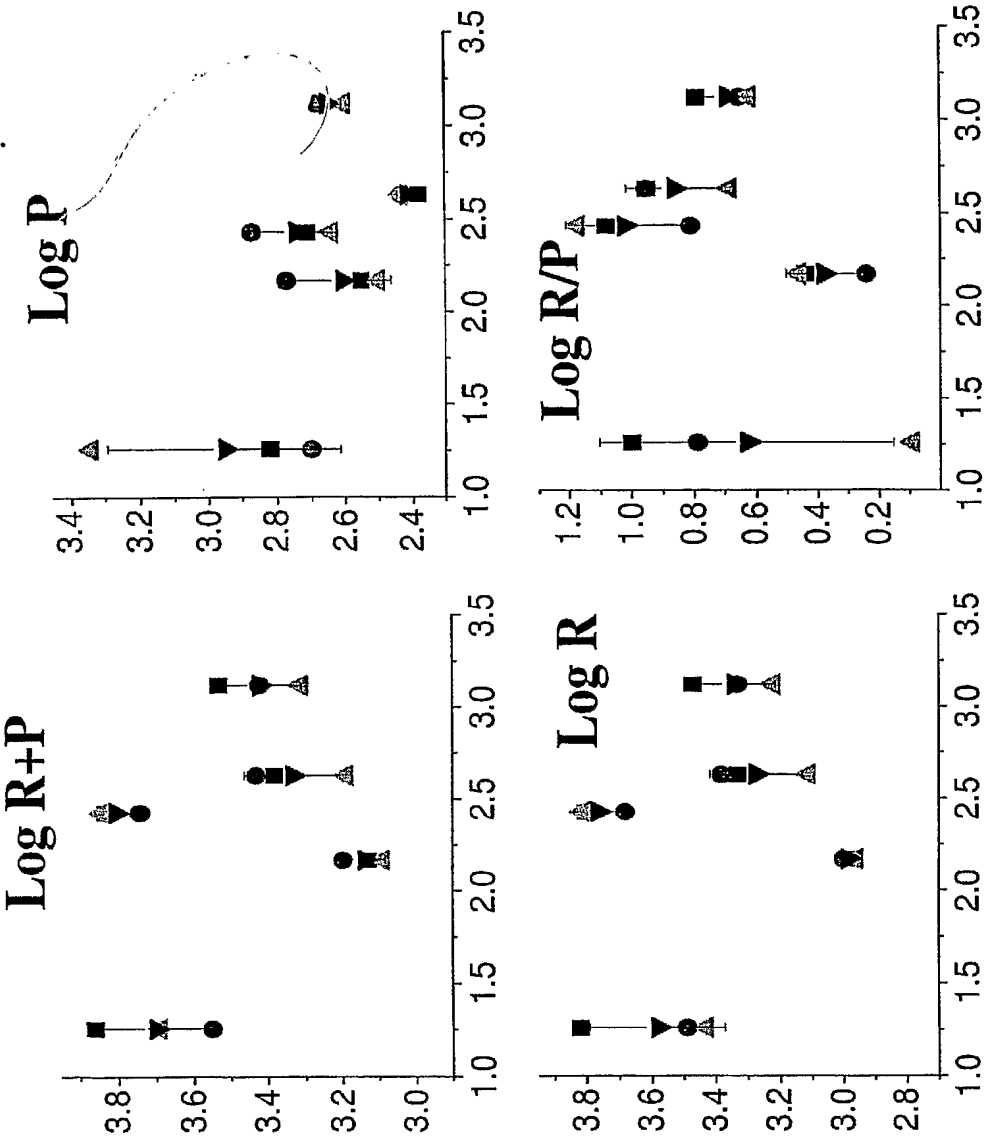


Figure 15

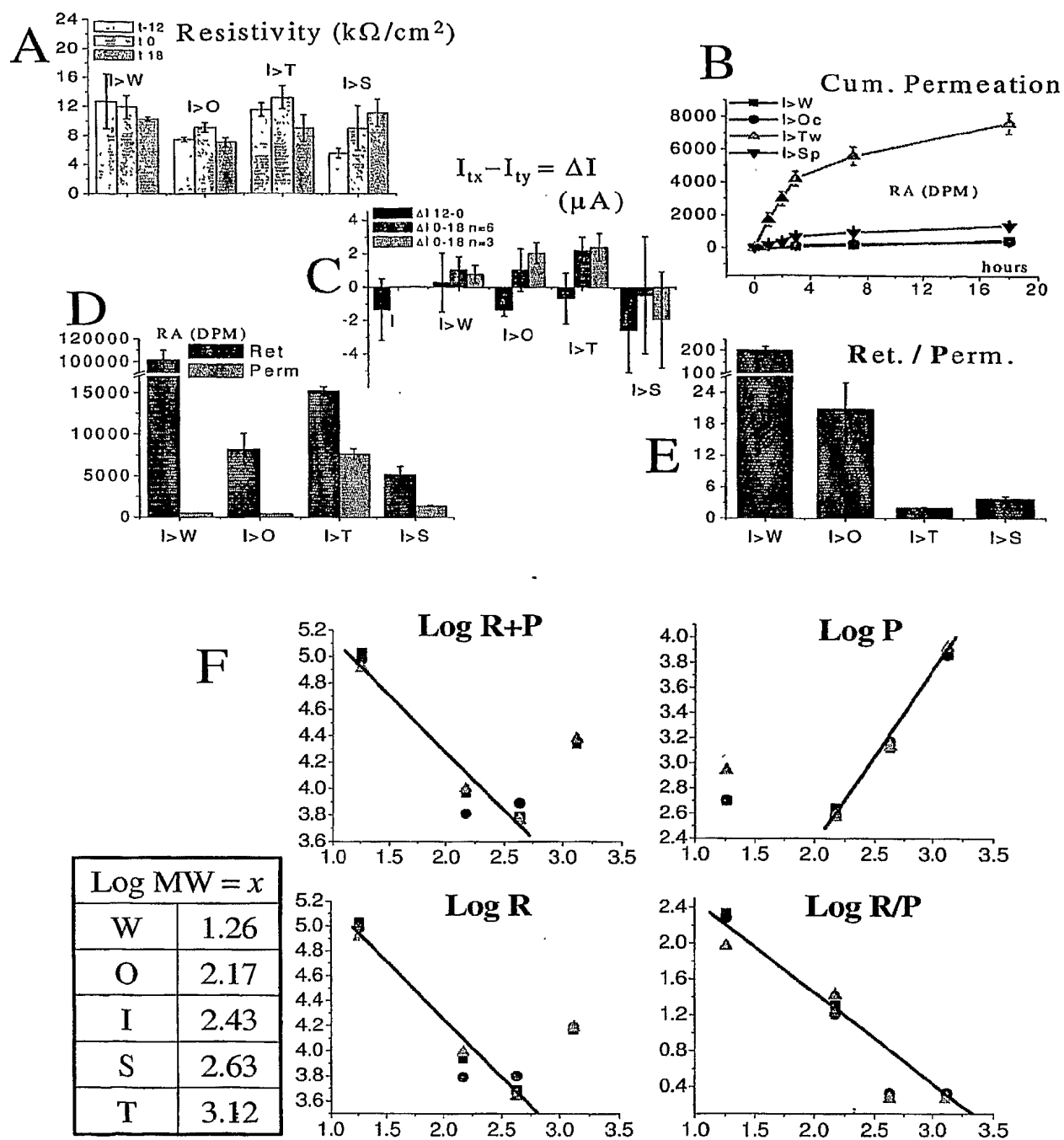


Figure 16

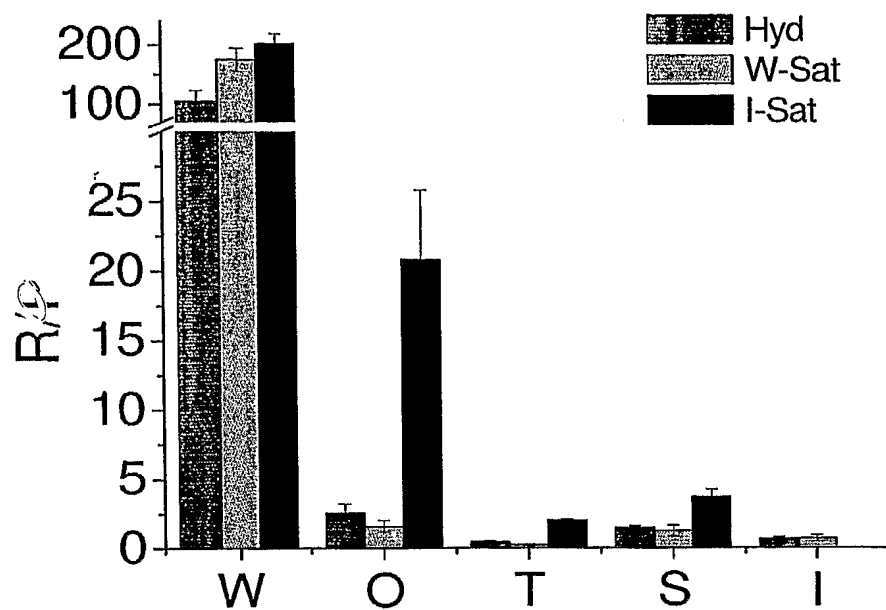


Figure 17

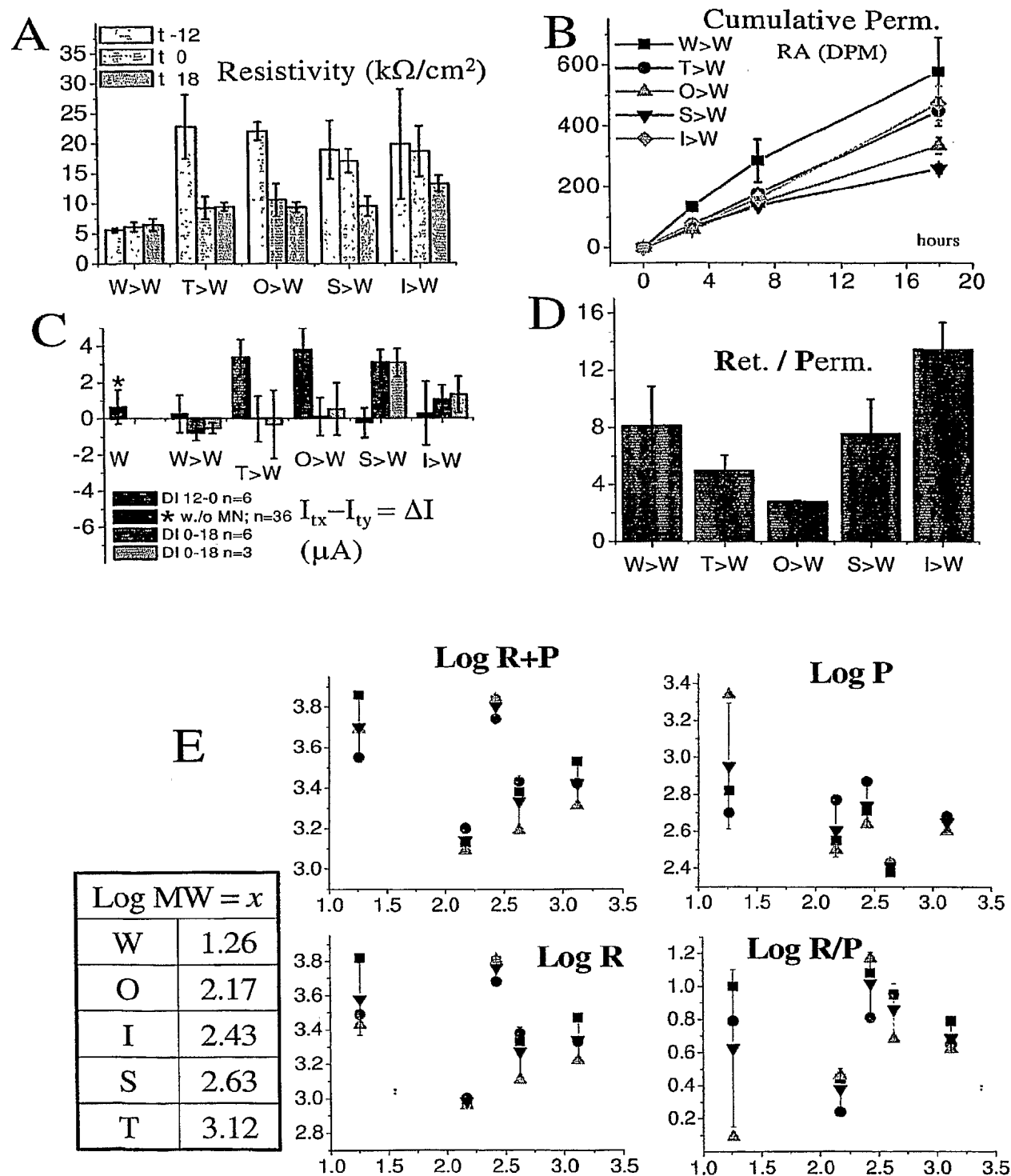


Figure 18

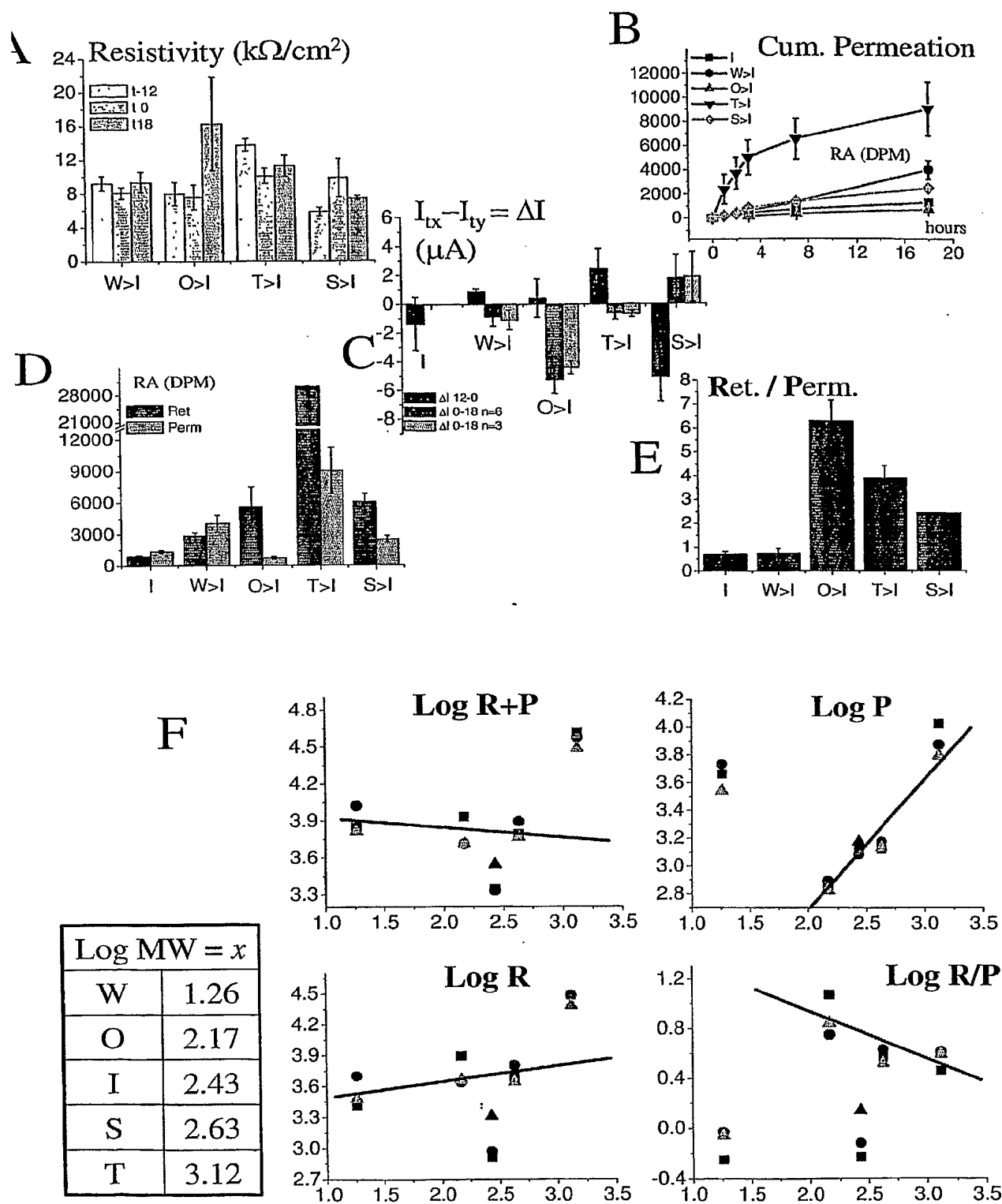


Figure 19

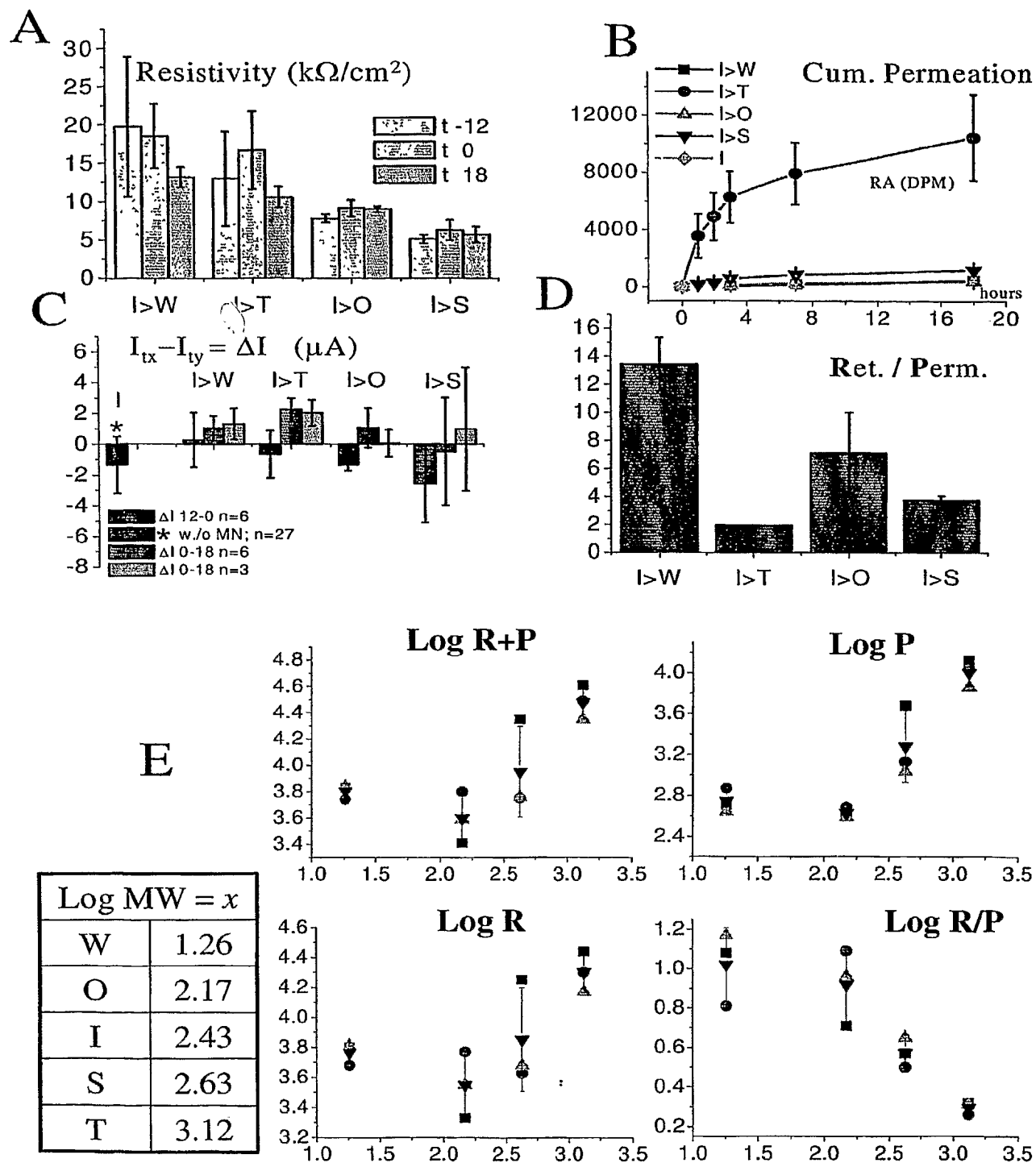


Figure 20

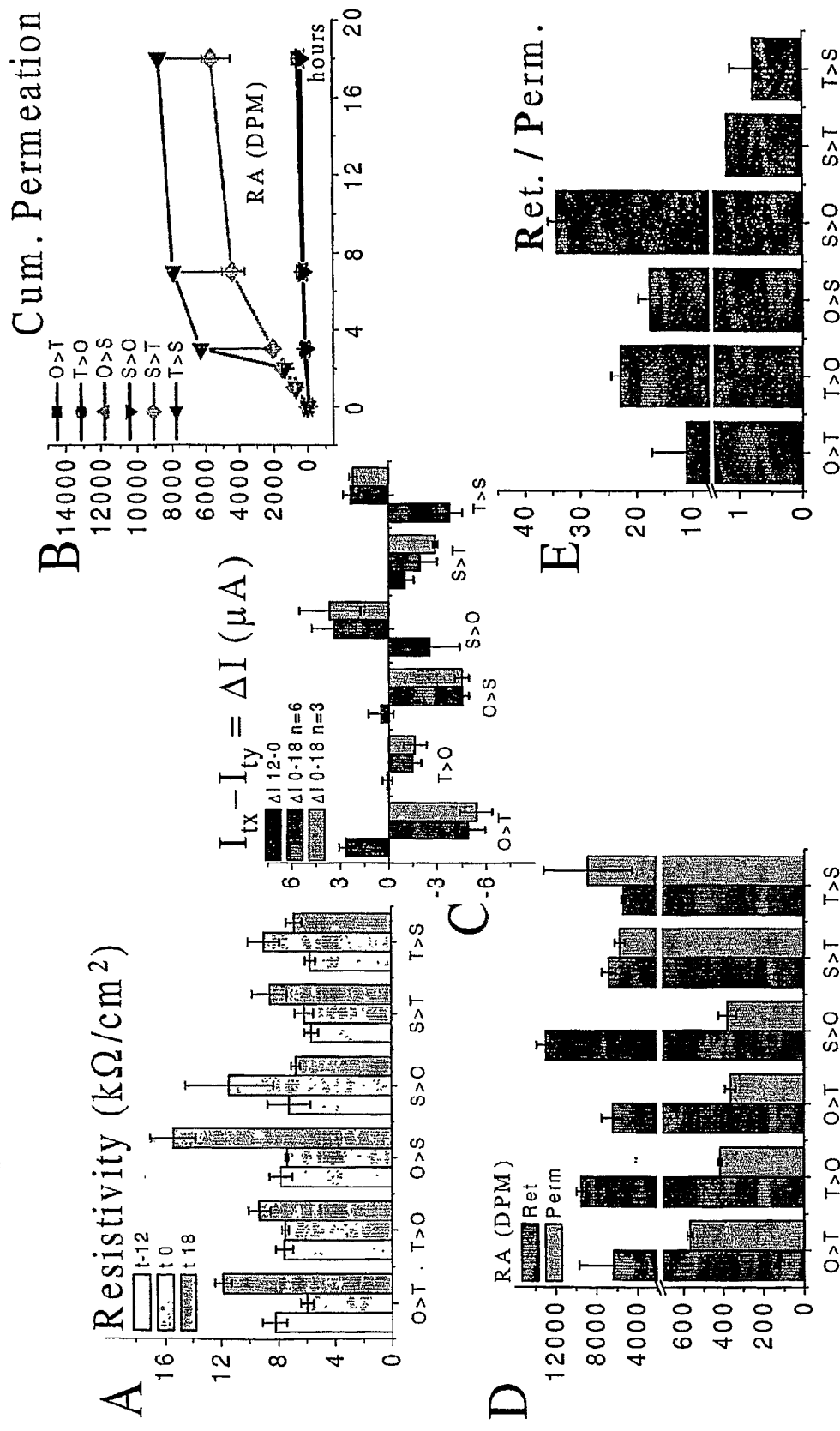


Figure 21

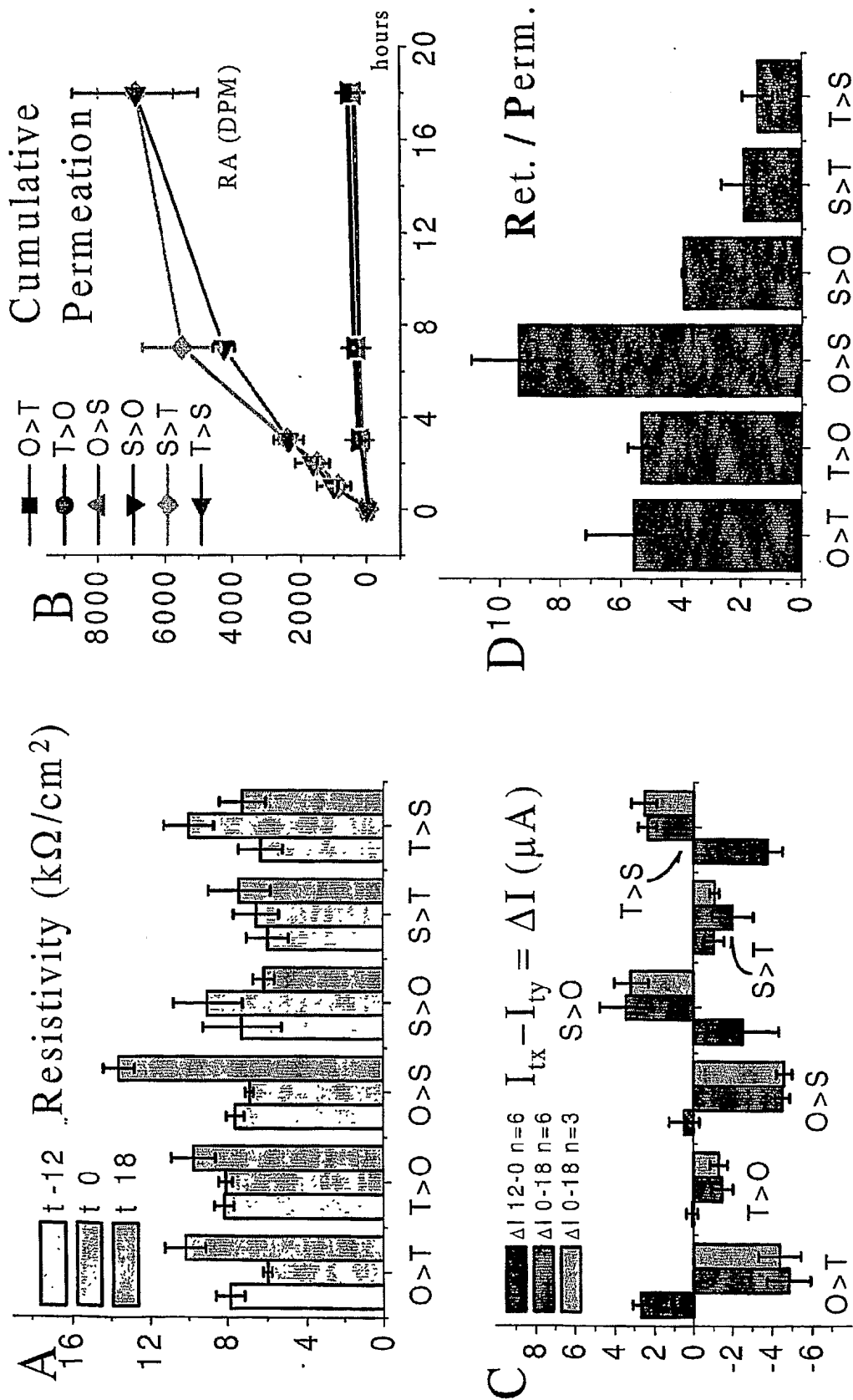


Figure 22



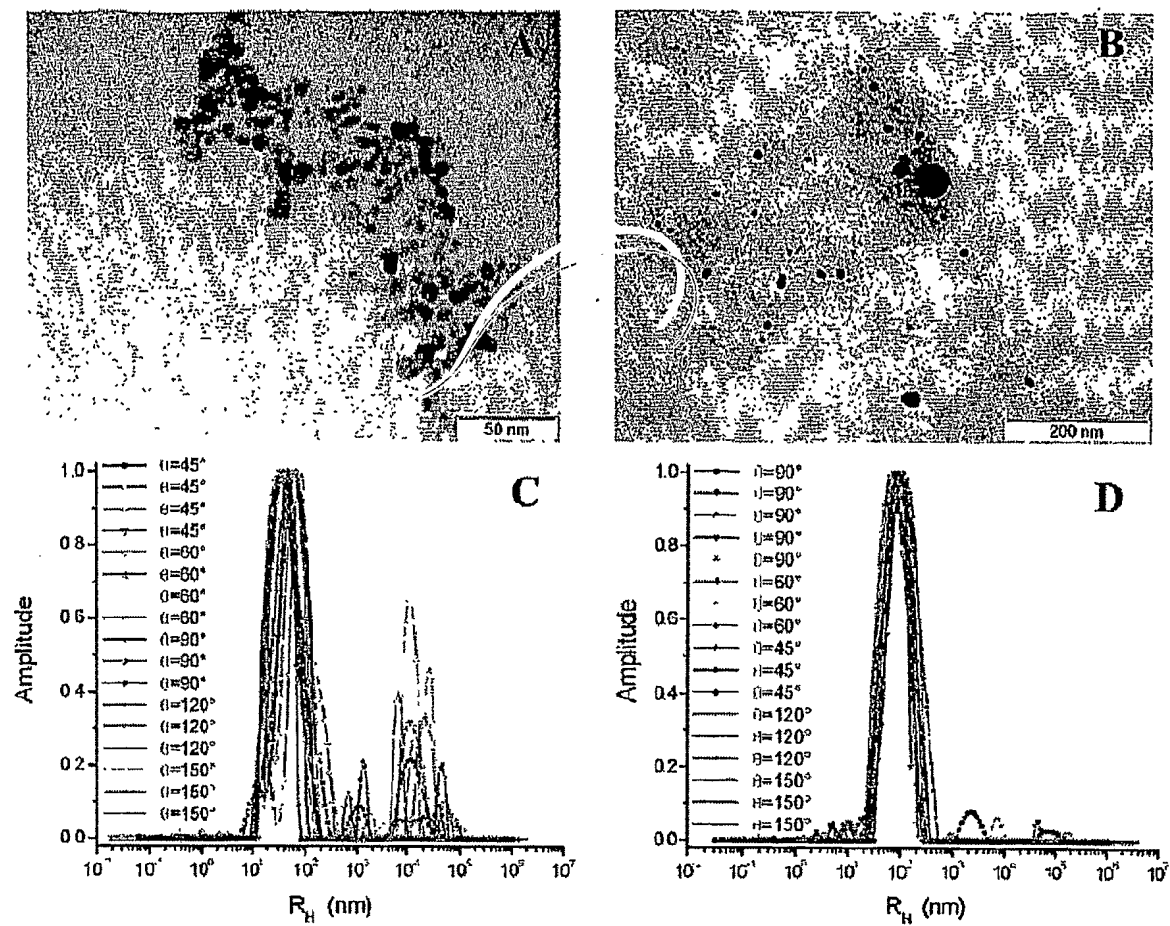


Figure 23

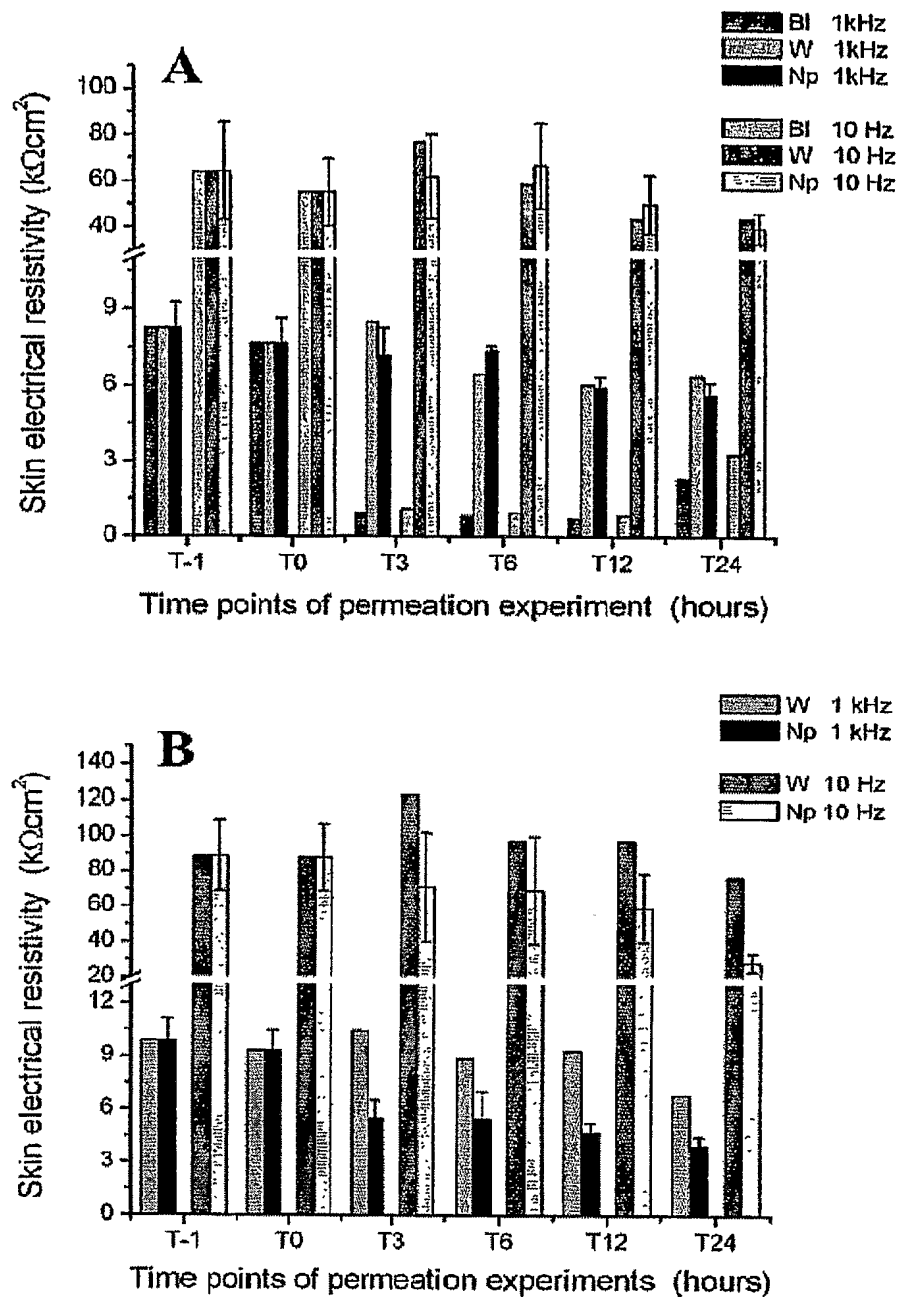


Figure 24

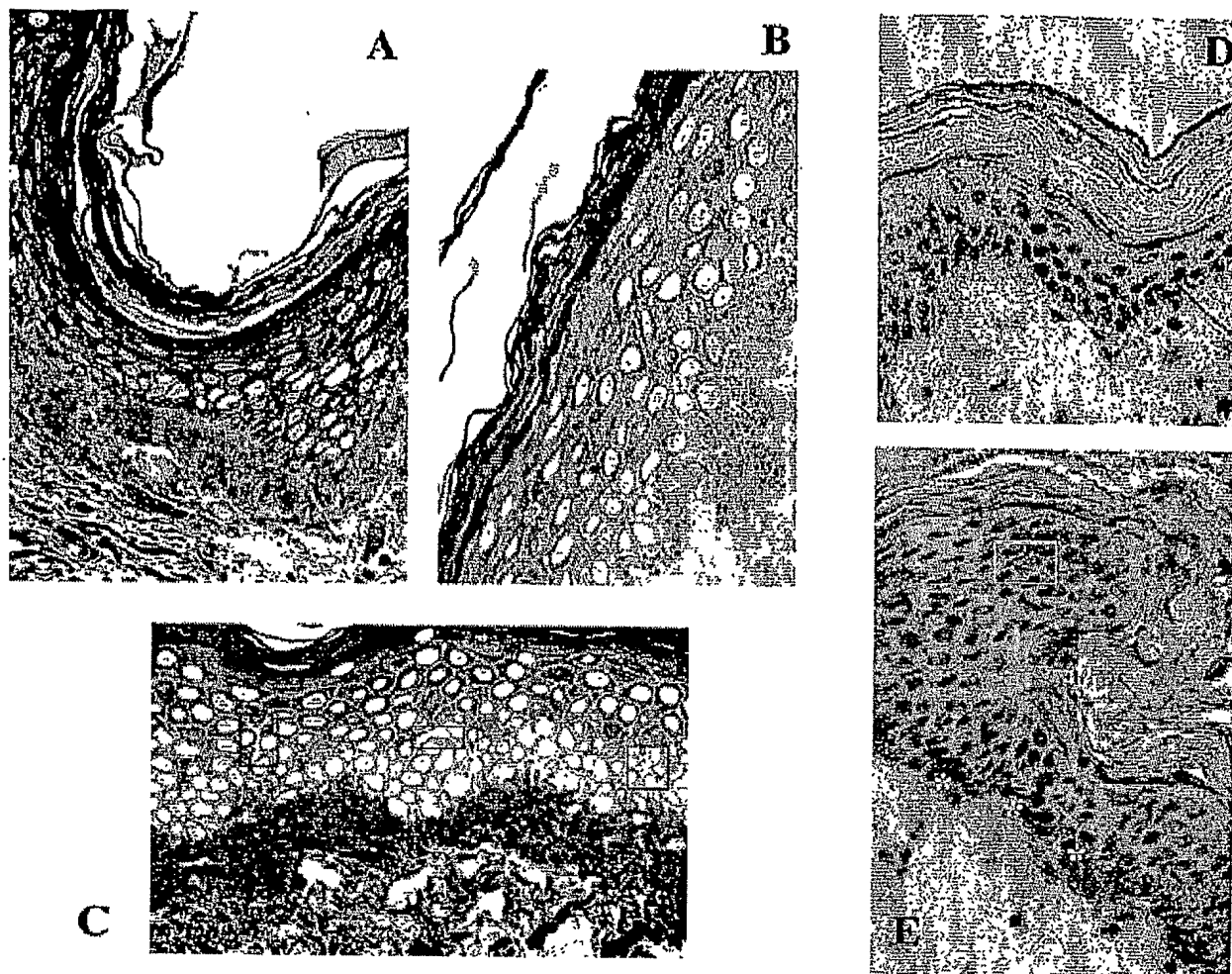


Figure 25

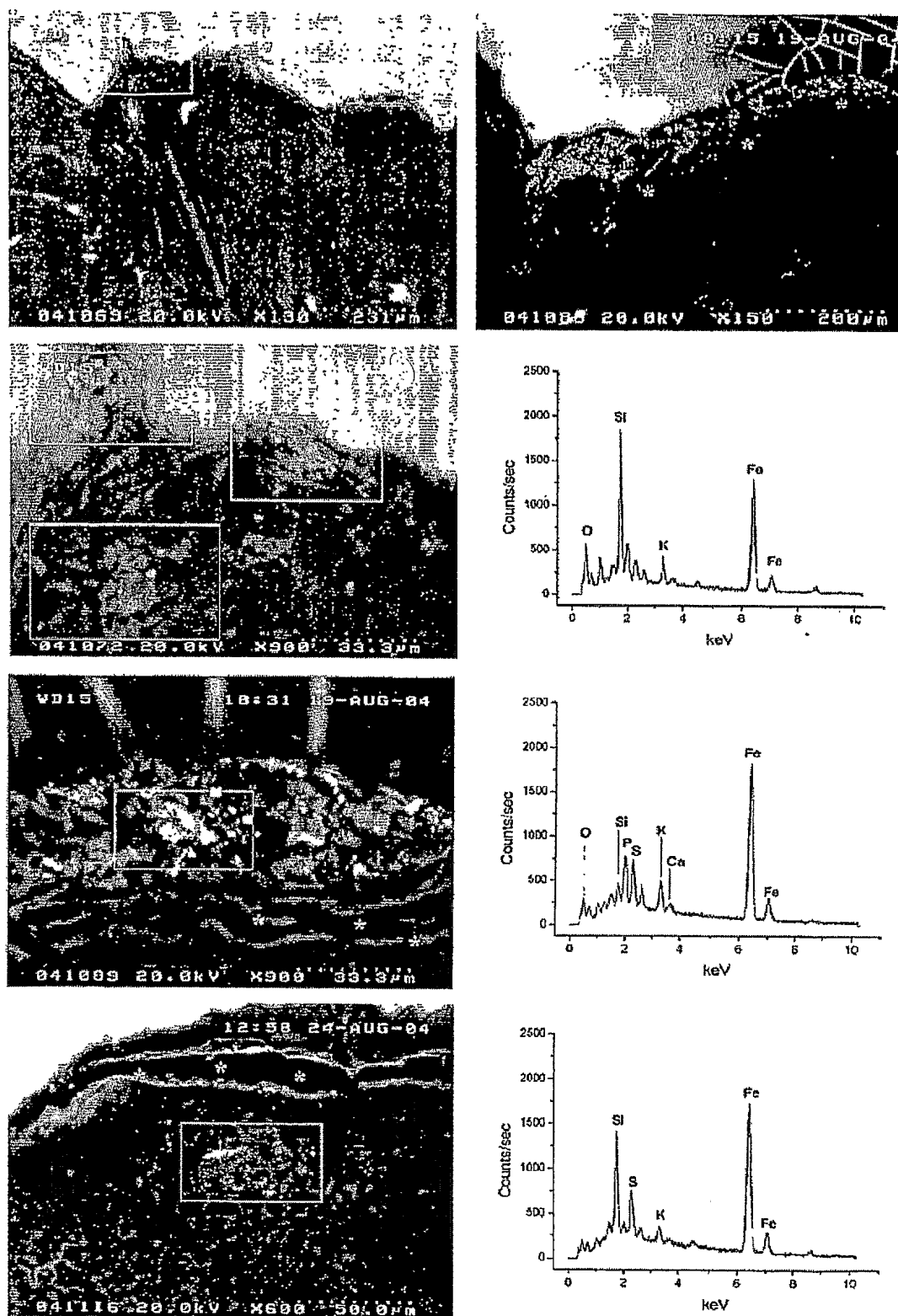


Figure 26